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(54) Title: METHODS AND COMPOUNDS FOR THE GENETIC TREATMENT OF HYPERLIPIDEMIA

(57) Abstract

The present invention concerns the introduction of specific alterations in the genes that encode three apolipoproteins, Apo A1, Apo B and Apo E. The alternations in Apo A1 introduce a cysteine residue so the disulfide cross-linked Apo A1 homodimers and Apo A1/A2 heterodimers can be formed. The alterations in Apo B introduce stop codons or frame shift mutations that cause the production of a truncated Apo B protein. The alterations in Apo E introduce specific point mutations that have been identified as protective. The production in the liver of a subject of these altered proteins reduces the risk of the subjects developing atherosclerosis. In one embodiment the genetic alterations are introduced by use of chimeric, mixed RNA/DNA, duplex oligonucleotides.

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METHODS AND COMPOUNDS FOR THE GENETIC TREATMENT OF HYPERLIPIDEMIA

This application claims benefit of the priority of application Serial No. 60/074,497, filed February 12, 1998 and application Serial No. 09/108,006 filed June 30, 1998.

1. FIELD OF THE INVENTION

The invention concerns methods and compositions for the use of recombinagenic oligonucleobases *in vivo* for the correction of disease causing genetic defects and the prevention of disease by introducing genetic modifications into the genes that encode Apolipoprotein B (Apo B), Apolipoprotein E (Apo E) and Apolipoprotein A1 (Apo A1).

2. BACKGROUND TO THE INVENTION

2.1 The Use of Chimeric Mutational Vectors to Effect Genetic Changes in Cultured Cells

The inclusion of a publication or patent application in this specification is not an admission that the publication or the invention, if any, of the application occurred prior to the present invention or resulted from the conception of a person other than the present inventors.

The published examples of recombinagenic oligonucleobases are termed Chimeric Mutational Vectors (CMV) or chimeraplasts because they contain both 2'-O-modified ribonucleotides and deoxyribonucleotides.

An oligonucleotide having complementary deoxyribonucleotides and ribonucleotides and containing a sequence homologous to a fragment of the bacteriophage M13mp19, was described in Kmiec, E.B., et al., November 1994, Mol. and Cell. Biol. **14**, 7163-7172. The oligonucleotide had a single contiguous segment of ribonucleotides. Kmiec et al. showed that the oligonucleotide was a substrate for the REC2 homologous pairing enzyme from *Ustilago maydis*.

Patent publication WO 95/15972, published June 15, 1995, and counterpart U.S. Patent No. 5,565,350 (the '350 patent) described duplex CMV for the introduction of genetic changes in eukaryotic cells. Examples in a *Ustilago maydis* gene and in the

murine ras gene were reported. The latter example was designed to introduce a transforming mutation into the ras gene so that the successful mutation of the ras gene in NIH 3T3 cells would cause the growth in soft agar of a colony of cells ("transformation"). The '350 patent reported that the maximum rate of transformation of NIH 3T3 was less than 0.1 %, i.e., about 100 transformants per 10^6 cells exposed to the ras duplex CMV. In the *Ustilago maydis* system, the rate of transformants was about 600 per 10^6. A chimeric vector designed to introduce a mutation into a human bcl-2 gene was described in Kmiec, E.B., February 1996, Seminars in Oncology **23**, 188.

A duplex CMV designed to repair the mutation in codon 12 of K-ras was described in Kmiec, E.B., December 1995, Advanced Drug Delivery Reviews **17**, 333-40. The duplex CMV was tested in Capan 2, a cell line derived from a human pancreatic adenocarcinoma, using LIPOFECTIN™ to introduce the duplex CMV into the Capan 2 cells. Twenty four hours after the duplex CMV was introduced, the cells were harvested and genomic DNA was extracted; a fragment containing codon 12 of K-ras was amplified by PCR and the rate of conversion estimated by hybridization with allele specific probes. The rate of repair was reported to be approximately 18%.

A duplex CMV designed to repair a mutation in the gene encoding liver/bone/kidney type alkaline phosphatase was reported in Yoon, K., et al., March 1996, Proc. Natl. Acad. Sci. **93**, 2071. The alkaline phosphatase gene was transiently introduced into CHO cells by a plasmid. Six hours later the duplex CMV was introduced. The plasmid was recovered at 24 hours after introduction of the duplex CMV and analyzed. The results showed that approximately 30 to 38% of the alkaline phosphatase genes were repaired by the duplex CMV.

WO 97/41411 and counterpart United States Patent No. 5,760,012 to E.B. Kmiec, A. Cole-Strauss and K. Yoon, and the publication Cole-Strauss, A., et al., September 1996, SCIENCE **273**, 1386 disclose duplex CMV that are used in the treatment of genetic diseases of hematopoietic cells, e.g., Sickle Cell Disease, Thalassemia and Gaucher Disease. United States Patent Application Serial No. 08/664,487, filed June 17, 1996, by E.B. Kmiec describes duplex CMV having non-natural nucleotides for use in specific, site-directed mutagenesis. The duplex CMV described in the applications and certain of the publications of Kmiec and his colleagues contain a central segment of

DNA:DNA homoduplex and flanking segments of RNA:DNA hybrid-duplex or 2'-OMe-RNA:DNA hybrid-duplex.

The work of Kmiec and his colleagues concerned cells that are mitotically active, i.e., proliferating cells, at the time they are exposed to CMV. Kmiec and colleagues used a CMV/liposomal macromolecular carrier complex in which the CMV were mixed with a pre-formed liposome or lipid vesicle. In such a complex the CMV are believed to adhere to the surface of the liposome.

Kren et al., June 1997, Hepatology **25**, 1462-1468, reported the successful use of a CMV in non-replicating, primary tissue-cultured rat hepatocytes to mutate the coagulation factor IX gene. Kren et al., March 1998, Nature Medicine **4**, 285 reported the use of a CMV *in vivo* to introduce a genetic defect in the same gene.

2.2 THE USE OF A POLYETHYLENIMINE MACROMOLECULAR CARRIER FOR *IN VIVO* AND *IN VITRO* TRANSFECTION

Branched chain polyethylenimine has been used as a carrier to introduce nucleic acids into eukaryotic cells both *in vivo* and *in vitro*. Boussif, O., et al., 1995, Proc. Natl. Acad. Sci. **92**, 7297; Abdallah, B. et al., 1996, Human Gene Therapy 7, 1947. Boletta, A., et al., 1997, **8**, 1243-1251. The *in vitro use of* galactosylated polyethylenimine to introduce DNA into cultured HepG2 hepatocarcinoma cell lines is reported by Zanta, et al., October 1, 1997, Bioconjugate Chemistry **8**, 839-844. The coupling of a protein ligand, transferrin, to polyethylenimine and its use to introduce a test gene into cultured cells by use of the transferrin receptor is described in Kircheis, R., et al., 1997, Gene Therapy **4**, 409-4-18. Branched chain polyethylenimines contain secondary and tertiary amino groups having a broad range of pK's and, consequently these polyethylenimines have a substantial buffering capacity at a pH where polylysine has little or no capacity, i.e., less than about 8. Tang, M.K., & Szoka, F.C., 1997, Gene Therapy **4**, 823-832. The use of branched chain polyalkanylimines, including polyethylenimine as carriers for the introduction of nucleic acids into cells is described in WO 96/02655 to J-P. Behr et al.

The successful *in vivo* and *in vitro* use of linear polyethylenimine to transfect a gene is reported by Ferrari, S., et al., 1997, Gene Therapy **4**, 1100-1106. Compositions

comprising a linear polyalkanylimine and a nucleic acid as disclosed in patent publication WO 93/20090 to S. Stein et al.

2.3 The Use of a Liposomal Carrier For *In Vivo* Transfection

The use of liposomes or lipid vesicles to introduce DNA encoding a foreign protein into cells has been described. The most frequently used techniques adhere the DNA to the surface of a positively charged liposome, rather than encapsulating the DNA, although encapsulated DNA techniques were known. United States Patent Nos. 4,235,871 and 4,394,448 are relevant. The field is reviewed by Smith, J.G., et al., 1993, Biochim. Biophy. Acta **1154**, 327-340 and Staubinger, R.M., et al., 1987, Methods in Enzymology **185**, 512. The use of DOTAP, a cationic lipid in a liposome to transfect hepatic cells *in vivo* is described in Fabrega, A.J., et al., 1996, Transplantation **62**, 1866-1871. The use of cationic lipid-containing liposomes to transfect a variety of cells of adult mice is described in Zhu, N., et al., 1993, Science **261**, 209. The use of phosphatidylserine containing lipids to form DNA encapsulating liposomes for transfection is described in Fraley, R., et al., 1981, Biochemistry **20**, 6978-87.

2.4 The Use of the Asialoglycoprotein Receptor for Hepatocellular Specific Transfection

United States Patent Nos. 5,166,320 and 5,635,383 disclose the transfection of hepatocytes by forming a complex of a DNA, a polycationic macromolecular carrier and a ligand for the asialoglycoprotein receptor. In one embodiment, the macromolecular carrier was polylysine. The use of a lactosylcerebroside containing liposome to transfect a hepatocyte *in vivo* is described by Nandi, P.K., et al., 1986, J. Biol. Chem. **261**, 16722-16722. The use of asialofetuin-labeled liposomes to transfect liver cells with a reporter plasmid is described in Hara et al., 1995, Gene Therapy **2**, 764-788. The use of galactosylated poyethyleneimine to transfect cultured hepatocytes is described in Zanta M-A., et al. abst. pub. Oct. 1, 1997, Bioconjugate Chem., **8**, 839-844.

2.5 Apo B100, Apo B48 and the Reduction of Serum LDL

Hepatic and Intestinal Lipoprotein Secretion: Both the liver and the intestines make and export lipoproteins for the transport of lipids. The lipoproteins are termed very low density lipoproteins (VLDL) and chylomicrons, respectively. VLDL and chylomicrons differ in size and in their major protein components. The major protein of VLDL is Apo B100, consisting of 4536 amino acids; the major protein of chylomicrons is Apo B48, which consists of the N-terminal 2152 amino acids of Apo B100. Apo B48 and Apo B100 are encoded by a single gene, the transcript of which is modified at nucleotide 6666 (codon 2179) by a sequence specific cytidine deaminase, termed apolipoprotein B mRNA editing enzyme (APOBE). The action of this enzyme converts a C to U and results in a stop codon.

Both VLDL, which contain Apo B100, and chylomicrons, which contain Apo B48 transport triglycerides in the vascular system to a delivery site. However, after triglyceride hydrolysis and delivery VLDL are transformed into LDL, while chylomicrons are not. High levels of circulating LDL *per se* and a high LDL:HDL ratio increase the risk of arterial atherosclerosis. Hence, it has been suggested that increasing the ratio of Apo B48 to Apo B100 would have a beneficial effect.

In many species of mammals, e.g., rats and mice, a high percentage of the lipid secretions of both liver and intestine contain Apo B48. Such species have markedly lower ratios of LDL:HDL. Greve J., et al., 1995, Proc. Zool. Soc., Calcutta, **47**, 93-100. In others, such as humans and rabbits, hepatocytes lack APOBE and the hepatocytes consequently produce only VLDL.

One strategy to reduce the atherosclerosis in humans has been to introduce the gene for the catalytic component of the apolipoprotein B editing enzyme (APOBEC-1) under the control of a constitutive promoter to convert Apo B100 transcripts into Apo B48 transcripts. The transient expression of APOBEC-1 in the hepatocytes of normal and genetically hyperlipidemic Watanabe rabbit does cause a transient reduction in the levels of LDL. Greeve, J., et al., 1996, J. Lipid Res. **37**, 2001-17. However, the uncontrolled production of APOBEC-1 is mutagenic and may cause hepatocellular hyperplasia and hepatocellular carcinoma. Yamanaka, S., et al., 1995, Proc. Natl. Acad. Sci. **92**, 8483-8487.

Individuals who are homozygous or mixed heterozygotes for genes encoding truncated Apo B100 have been observed. Malloy et al., 1981, J. Clin. Invest. **67,** 1441; Hardman, D.A., et al., 1991, J. Clin. Invest. **88,** 1722. These individuals have low or absent LDL. For example, deletion of nucleotides 5391-5394 results in a frame shift mutation and a shortened Apo B (B37). These patients are most often asymptomatic. Steinberg, D., et al., 1979, J. Clin. Invest. **64,** 292; Young, S.G., et al., 1988, Science **241,** 591; Young, S.G., 1987, J. Clin. Invest. **79,** 1831. Reviewed Linton, M.F., 1993, J. Lipid. Res. **34,** 521; Kane, J.P. & Havel, R.J., 1995, Chapt. 57, THE METABOLIC BASIS OF INHERITED DISEASE, ed. Scriver et al. (McGraw Hill, New York). Similarly, as many as 1 in every 3,000 persons has a serum cholesterol level of 100 mg/dl or less because the individual is heterozygous for a truncated Apo B gene. *Ibid.*, p. 1866.

Truncations that result in an Apo B that are shorter than Apo B 31 do not circulate. Truncated Apo B 86, 87 and 90 have been observed. Apo B 86 and Apo B 87, are not associated with LDL while Apo B 90 is. Each mutation is associated with hypobetalipoproteinemia. Linton, M.L., et al., 1990, Clin. Res. **38,** 286A (abstr.); Tennyson, G.E., et al., 1990, Clin. Res. **38,** 482A (abstr.); Kruhl, E.S., et al., 1989, Arteriosclerosis **9,** 856.

2.6 APO E POLYMORPHISM AND TYPE III HYPERLIPIDEMIA

Apolipoprotein E is the major ligand for the LDL receptor for lipoproteins that contain Apo B48. There are three allelic forms of human Apo E that differ from each other by one or two amino acids: Apo E2 (Cys^{112} Cys^{158}); Apo E3 (Cys^{112} Arg^{158}); and Apo E4 (Arg^{112} Arg^{158}). There is considerable geographical variation in the prevalences of the alleles. Excluding Africa, E2 ranges between 4% and 12 %, E3 between 70% and 85% and E4 between 7.5 and 25%. In the Sudan, the prevalences are 8.1%, 61.9% and 29.1%, respectively. Mahley, R.W. & Rall, S.C., Jr., 1995, Chapt. 61, THE METABOLIC BASIS OF INHERITED DISEASE, ed. Scriver et al. (McGraw Hill, New York). Thus approximately 1% of the North American and European population are Apo E 2/2 homozygotes. Of these homozygotes approximately between 2% and 10% display type III hyperlipidemia. Paradoxically, however, Apo E 2/2 homozygotes that have not

developed overt Type III hyperlipidemia display lower than average LDL associated cholesterol. Davignon, J., 1988, Arteriosclerosis **8,** 1.

The E4 allele is also associated with increased incidence of a major disease, Alzheimer's Disease, and with increased risk of coronary artery disease. Roses, A.D., 1996, Ann. NY Acad. Sci. **802,** 50-57; Okumoto, K., & Fujiki, Y., 1997, Nature Genetics **17,** 263; Kuusi, T., et al., 1989, Arteriosclerosis **9,** 237. A polymorphism in the region 491 nt 5' to the transcription start site of the Apo E gene is also an independently associated with increased risk of Alzheimer's disease. Individuals homozygous for the -491-A genotype have an increased risk of Alzheimer's, while individuals homozygous or heterozygous for the -491 T genotype have no increased risk. Bullido, M.J., 1998, et al., Nature Genetics **18,** 69-71.

The E2 allele in most individuals is associated with the lowest levels of serum cholesterol and LDL. However, about 5% of E2/E2 homozygous persons who are subject to environmental or genetic stress develop type III hyperlipidemia. The most common stressors are hypothyroidism, untreated diabetes mellitus, alcoholism and marked weight gain. Removal of the stressor usually results in control of the hyperlipidemia. Rare patients with type III hyperlipidemia have mutant Apo E genes. Mahley & Rall, *ibid.* Table 61-5.

2.7 APO A1 AND HDL

High density lipoproteins (HDL) transport cholesterol and phospholipids from peripheral extraheptatic locations to the liver. In particular, HDL are believed to remove lipid deposits from vascular endothelial cells and that the observed negative correlation between the levels of HDL-cholesterol (HDL-C) and coronary artery disease is due to this function. Eisenberg, S., 1984, J. Lipid Research **25,** 1017; Gordon, D.J., et al., 1986, Circulation **74,** 1217. HDL are secreted by the liver and intestines as nacent HDL particles containing four molecules of apo A1, which is a 243 amino acid protein. The nascent HDL physically attract free cholesterol from cell membranes and/or other lipoproteins. The resulting particle contains apo A1, phospholipid, and cholesterol. Such particles are substrates for lecithin:cholesterol acyltransferase (LCAT), which esterifies the free cholesterol to cholesterol esters. The presence of the more hydrophobic cholesterol

esters transforms the nascent HDL initially to a more stable mature HDL3 and subsequently HDL3 particle.

Cholesterol ester transfer protein removes the esterified cholesterol from the HDL3 and HDL2 particles and transfers them into LDL and thence into hepatocytes through the LDL receptors.

Two mutations of apo A1 have been discovered wherein the levels of HDL are depressed. The mutations are missense mutations that replace an arginine with a cysteine amino acid. Weisgraber, K.H., et al., 1983, J. Biol. Chem. **258,** 2508 (apo A1 milano $(Arg\rightarrow Cys)^{173}$); Bruckert, E., et al., 1997, Atherosclerosis **128,** 121 (apo A1 R151C $(Arg\rightarrow Cys)^{151}$). The mutation is dominant, i.e., HDL levels are depressed in affected individuals who are heterozygous for the mutation. An explanation for this is that the mutant apo A1 proteins form cystine linked heterodimers with wild apo A2 molecules, which are a minor apolipoprotein found in HDL, as well as homodimers.

Surprising even though the lipid profile, i.e., low HDL and elevated triglycerides, is one that ordinarily is associated with accelerated atherosclerosis, the mutations are not associated with atherosclerosis, but, rather, are believed to be protective of atheroscelerosis.

The mechanism of protection is not established. A large (N = 33) retrospective epidemologic study of apo A1 milano carriers shows that there is a low incidence of coronary artery disease in these individuals, however, the significance of this fact must be viewed in the context of similar low incidence among non-affected residents of the village Limone sul Garda. Gualandri, V., et al., 1985, Am. J. Hum.Gen. **37,** 1083. *In vitro* studies indicate that the mutant apo A1 is not more effective in recruiting plasma membrane cholesterol. Bielicki, J.K., et al., 1997, Arterioscler Thromb Vasc Biol **17,** 1637 There is direct evidence that the administration of exogenous apo A1 milano, but not wild type apo A1, in the form of a complex with phospholipids, is protective in an accepted model system of atherosclerosis. Ameli, S. et al., 1994, Circulation **90,** 1935; Shah, P.K., et al., 1998, Circulation **97,** 780.

3. SUMMARY OF THE INVENTION

The present invention concerns methods of treatment and/or prophylaxis which consists of the introduction of specific genetic alterations in genes of a subject individual. In one embodiment, the specific genetic alteration blocks the synthesis of Apo B100 and thereby reduces the level of LDL cholesterol. In an alternative embodiment, the specific alteration converts an Apo E4 allele to an Apo E3 or Apo E2 allele, which is associated with decreased risk of atherosclerosis and Alzheimer's Disease. In further alternative embodiments, the invention concerns the correction of inherited genetic defects in the genes of hepatocytes of individuals having a disease caused by such defects.

The present invention further comprises a method of treating and/or preventing atherosclerosis by causing mutations in the genes encoding apo A1 and compounds that are useful for the introduction of such mutations. The mutations useful for the practice of the invention are mutations that insert a cysteine residue that forms a cystine and results in the formation of homodimers and apo A2-containing heterodimers.

The embodiments of the invention can be practiced using any oligonucleotide or analog or derivative thereof, now known or hereafter developed, that can cause specific genetic alterations in the genome of the hepatocytes of the subject individual (hereafter a "recombinagenic oligonucleobase"), for example a chimeric mutational vector (CMV) as, for example, described in United States patent No. 5,565,350, No. 5,731,181, and No. 5,760,012. Alternatively, the recombinagenic oligonucleobase can be a heteroduplex mutational vector or a non-chimeric mutational vector as described in U.S. patent application No. 09/078,063 and No. 09/078,064, filed May 12, 1998, each of which are hereby incorporated by reference.

In a preferred embodiment the recombinagenic oligonucleobase is complexed with a macromolecular carrier to which is attached a specific ligand. The ligand is selected to bind to a cell-surface receptor that is internalized into hepatocytes through clathrin-coated pits into endosomes. The cell surface receptors that bind such ligands are termed herein "clathrin-coated pit receptors". Examples of hepatic clathrin-coated pit receptors include the low density lipoprotein (LDL) receptor and the asialoglycoprotein receptor.

In specific embodiments the macromolecular carrier can be 1) an aqueous-cored lipid vesicle of between 25 nm and 400 nm diameter, wherein the aqueous core contains the CMV; 2) a lipid nanosphere of between 25 nm and 400 nm diameter, having a lipid core, wherein the lipid core contains a lipophilic salt of the CMV; or 3) a polycationic salt of the CMV. Examples of polycations for such salts include polyethylenimine, polylysine and histone H1. In one embodiment the polycation is a linear polyethylenimine (PEI) salt having a mass average molecular weight greater than 500 daltons and less than 1.3 Md. Alternatively the polycation can be a branched-chain polyethylenimine.

4. BRIEF DESCRIPTION OF THE FIGURE

Figure 1 is a schematic of one embodiment of CMV useful in the invention.

Figures 2A-2C show the genomic sequence of human APO E gene with translation of exons. Introns are in lower case and exons are in upper case.

Figure 3A-3G shows the sequence of the human APO A1 gene (SEQ ID No. 59)

5. DEFINITIONS

The invention is to be understood in accordance with the following definitions.

An oligonucleobase is a polymer of nucleobases, which polymer can hybridize by Watson-Crick base pairing to a DNA having the complementary sequence.

Nucleobases comprise a base, which is a purine, pyrimidine, or a derivative or analog thereof. Nucleobases include peptide nucleobases, the subunits of peptide nucleic acids, and morpholine nucleobases as well as nucleosides and nucleotides. Nucleosides are nucleobases that contain a pentosefuranosyl moiety, e.g., an optionally substituted riboside or 2'-deoxyriboside. Nucleosides can be linked by one of several linkage moieties, which may or may not contain a phosphorus. Nucleosides that are linked by unsubstituted phosphodiester linkages are termed nucleotides.

An oligonucleobase chain has a single 5' and 3' terminus, which are the ultimate nucleobases of the polymer. A particular oligonucleobase chain can contain nucleobases of all types. An oligonucleobase compound is a compound comprising one or more oligonucleobase chains that are complementary and hybridized by Watson-Crick base pairing. Nucleobases are either deoxyribo-type or ribo-type. Ribo-type nucleobases are

pentosefuranosyl containing nucleobases wherein the 2' carbon is a methylene substituted with a hydroxyl, alkyloxy or halogen. Deoxyribo-type nucleobases are nucleobases other than ribo-type nucleobases and include all nucleobases that do not contain a pentosefuranosyl moiety.

An oligonucleobase strand generically includes both oligonucleobase chains and segments or regions of oligonucleobase chains. An oligonucleobase strand has a 3' end and a 5' end. When a oligonucleobase strand is coextensive with a chain, the 3' and 5' ends of the strand are also 3' and 5' termini of the chain.

A region is a portion of an oligonucleobase, the sequence of which is derived from some particular source, e.g., a CMV having a region of at least 15 nucleotides having the sequence of a fragment of the human ß-globin gene. A segment is a portion of a CMV having some characteristic structural feature. A given segment or a given region can contain both 2'-deoxynucleotides and ribonucleotides. However, a ribo-type segment or a 2'-deoxyribo-type segment contain only ribo-type and 2'-deoxyribo-type nucleobases, respectively.

6. DETAILED DESCRIPTION OF THE INVENTION

6.1 THE STRUCTURE OF THE CHIMERIC MUTATIONAL VECTOR

The Chimeric Mutational Vectors (CMV) are comprised of oligonucleobases, i.e., polymers of nucleobases, which polymers form Watson-Crick base pairs of purines and pyrimidines (hybridize), to DNA having the appropriate sequence. Each CMV is divided into a first and a second strand of at least 15 nucleobases each that are complementary to each other. The strands can be, but need not be, covalently linked. Nucleobases contain a base, which is either a purine or a pyrimidine or analog or derivative thereof. There are two types of nucleobases. Ribo-type nucleobases are ribonucleosides having a 2'-hydroxyl, substituted 2'-hydroxyl or 2'-halo-substituted ribose. All nucleobases other than ribo-type nucleobases are deoxyribo-type nucleobases. Thus, deoxy-type nucleobases include peptide nucleobases. As used herein, only a recombinagenic oligonucleobase that contains at least three contiguous ribo-type nucleobases that are hybridized to deoxyribo-type nucleobases are considered CMV.

The sequence of the first and second strands consists of at least two regions that are homologous to the target gene, i.e., have the same sequence as fragments of the target gene, and one or more regions (the "mutator regions") that differ from the target gene and introduce the genetic change into the target gene. The mutator region is located between homologous regions. In certain embodiments of the invention, each of the flanking homologous regions contains a ribo-type segment of at least three ribo-type nucleobases, that form a hybrid duplex, preferably at least six ribo-type nucleobases and more preferably at least ten ribo-type nucleobases in length, but not more than 25 and preferably not more than 20, more preferably not more than 15 ribo-type nucleobases. The hybrid-duplex-forming ribo-type oligonucleobase segments need not be adjacent to the mutator region. In certain embodiments of the invention the ribo-type oligonucleobase segments are separated from the mutator region by a portion of the homologous region comprising deoxyribo-type nucleobases. In these embodiments the mutator region is also composed of deoxyribo-type nucleobases. Accordingly, the mutator region and a portion of one or both homologous regions form an intervening segment of homo-duplex, which separates the two segments of hybrid-duplex.

The total length of all homologous regions is preferably at least 16 nucleobases and is more preferably from about 20 nucleobases to about 60 nucleobases in length.

Preferably, the mutator region consists of 20 or fewer bases, more preferably 6 or fewer bases and most preferably 3 or fewer bases. The mutator region can be of a length different than the length of the sequence that separates the regions of the target gene homology with the homologous regions of the CMV so that an insertion or deletion of the target gene results. When the CMV is used to introduce a deletion in the target gene there is no base identifiable as within the mutator region. Rather, the mutation is effected by the juxtaposition of the two homologous regions that are separated in the target gene. For the purposes of the invention, the length of the mutator region of a CMV that introduces a deletion in the target gene is deemed to be the length of the deletion. In one embodiment the mutator region is a deletion of from 6 to 1 bases or more preferably from 3 to 1 bases. Multiple separated mutations can be introduced by a single CMV, in which case there are multiple mutator regions in the same CMV. Alternatively multiple CMV can be used simultaneously to introduce multiple genetic changes in a single gene

or, alternatively to introduce genetic changes in multiple genes of the same cell. Herein the mutator region is also termed the heterologous region.

In one embodiment the CMV is a single oligonucleobase chain of between 40 and 100 nucleobases. In an alternative embodiment, the CMV comprises a first and a second oligonucleobase chain, each of between 20 and 100 bases; wherein the first chain comprises the first strand and the second chain comprises the second strand. The first and second chains can be linked covalently by other than nucleobases or, alternatively, can be associated only by Watson-Crick base pairings. In an alternative embodiment the CMV is a first strand which is a single oligonucleobase chain and a second strand, complementary to the first which consists of two oligonucleobase chains, which are linked to the first strand chain by linkers. The combined length of the two chains of the second strand is the length of the first strand.

Linkers: Covalent linkage of the first and second strands can be made by oligo-alkanediols such as polyethyleneglycol, poly-1,3-propanediol or poly-1,4-butanediol. The length of various linkers suitable for connecting two hybridized nucleic acid strands is understood by those skilled in the art. A polyethylene glycol linker having from six to three ethylene units and terminal phosphoryl moieties is suitable. Durand, M. et al., 1990, Nucleic Acid Research **18,** 6353; Ma, M. Y-X., et al., 1993, Nucleic Acids Res. **21,** 2585-2589. A preferred alternative linker is bis-phosphorylpropyl-trans-4,4'-stilbenedicarboxamide. Letsinger, R.L., et alia, 1994, J. Am. Chem. Soc. **116,** 811-812; Letsinger, R.L. et alia, 1995, J. Am. Chem. Soc. **117,** 7323-7328, which are hereby incorporated by reference. Such linkers can be inserted into the CMV using conventional solid phase synthesis. Alternatively, the strands of the CMV can be separately synthesized and then hybridized and the interstrand linkage formed using a thiophoryl-containing stilbenedicarboxamide as described in patent publication WO 97/05284, February 13, 1997, to Letsinger R.L. et alia.

In a further alternative embodiment the linker can be a single strand oligonucleobase comprised of nuclease resistant nucleobases, e.g., a 2'-O-methyl, 2'-O-allyl or 2'-F-ribonucleotides. The tetranucleotide sequences TTTT, UUUU and UUCG and the trinucleotide sequences TTT, UUU, or UCG are particularly preferred nucleotide linkers.

Nucleotides: In an alternative embodiment the invention can be practiced using CMV comprising deoxynucleotides or deoxynucleosides and 2'-O substituted ribonucleotides or ribonucleosides. Suitable substituents include the substituents taught by the Kmiec Application, C_{1-6} alkane. Alternative substituents include the substituents taught by U.S. Patent No. 5,334,711 (Sproat) and the substituents taught by patent publications EP 629 387 and EP 679657 (collectively, the Martin Applications), which are hereby incorporated by reference. As used herein a 2' fluoro, chloro or bromo derivative of a ribonucleotide or a ribonucleotide having a substituted 2'-O as described in the Martin Applications or Sproat is termed a "2'-Substituted Ribonucleotide." Particular preferred embodiments of 2'-Substituted Ribonucleotides are 2'-fluoro, 2'-methoxy, 2'-propyloxy, 2'-allyloxy, 2'-hydroxylethyloxy, 2'-methoxyethyloxy, 2'-fluoropropyloxy and 2'-trifluoropropyloxy substituted ribonucleotides. In more preferred embodiments the 2'-Substituted Ribonucleotides are 2'-fluoro, 2'-methoxy, 2'-methoxyethyloxy, and 2'-allyloxy substituted nucleotides.

2'-Substituted Ribonucleosides are defined analogously. Particular preferred embodiments of 2'-Substituted Ribonucleosides are 2'-fluoro, 2'-methoxy, 2'-propyloxy, 2'-allyloxy, 2'-hydroxylethyloxy, 2'-methoxyethyloxy, 2'-fluoropropyloxy and 2'-trifluoropropyloxy substituted ribonucleotides. In more preferred embodiment on the 2'-Substituted Ribonucleosides are 2'-fluoro, 2'-methoxy, 2'-methoxyethyloxy, and 2'-allyloxy substituted nucleotides.

The term "nuclease resistant ribonucleoside" encompasses 2'-Substituted Ribonucleosides, including 2'-Substituted Ribonucleotides and also all 2'-hydroxyl ribonucleosides other than ribonucleotides. In a preferred embodiment , the CMV preferably includes at least three and more preferably six nuclease resistant ribonucleosides. In one preferred embodiment the CMV contains no nuclease sensitive ribonucleosides. In an alternative preferred embodiment, every other ribonucleoside is nuclease resistant. Certain 2'-blocking groups can be more readily synthesized for purines or pyrimidines. In one embodiment of the CMV only the ribonucleoside purines or only the ribonucleoside pyrimdines are nuclease resistant.

Recombinagenic oligonucleobases, including non-chimeric mutational oligonucleobases and improved CMV and their use in eukaryotic cells and cell-free

systems are described in U.S. patent applications Serial No. 09/078,063, filed May 12, 1998, and Serial No. 09/078,064, filed May 12, 1998, which are each hereby incorporated in their entirety. These mutational oligonucleobases can be used in the same manner as the CMV described in this application.

6.2 The Gene-Specific Structure of the Chimeric Mutational Vector

Figure 1 shows a diagram of a CMV according to one embodiment of the invention. In the Figure segments "a" and "c-e" are target gene specific segments of the CMV. The sequence of segment "a" and "c-e" are complements of each other. The sequence of segments "f" and "h" are also complements of each other but are unrelated to the specific target gene and are selected merely to ensure the stability of hybridization in order to protect the 3' and 5' ends. Additional protection of the 3' and 5' ends can be accomplished by making the 5' and 3' most internucleotide bonds a phosphorothioate, phosphonate or any other nuclease resistant bond. The sequence of segments "f" and "h" can be 5'-GCGCG-3' or permutations thereof. Segments "g" and "b" can be any linker that covalently connects the two strands, e.g., four unpaired nucleotides or an alkoxy oligomer such as polyethylene glycol. When segments "g" and "b" are composed of other than nucleobases, then segments "a", "c-f" and "h" are each an oligonucleobase chain.

The ribo-type nucleobase segments are segments "c" and "e," which form hybrid-duplexes by Watson-Crick base pairing to the complementary portions of segment "a." The segment "a" can have the sequence of either the coding or non-coding strand of the gene.

Table I contains SEQ ID No. 4 - No. 21 and Table III contains SEQ ID No. 22-25 and 54-58, which are examples of the sequences that can be used to practice the invention. The mutator region in each case is underlined and in bold. CMV having a segment "a" with a sequence selected from the sequences of Table I can be used to practice the invention. Alternatively, segment "a" may have the sequence of the complement of a sequence of Table I. As used herein, a CMV or other type of recombinagenic oligonucleobase comprises a sequence if either strand of the CMV or recombinagenic oligonucleobase comprises the sequence or comprises a sequence containing ribo-type nucleobases with uracil bases replacing thymine bases. Thus, for

example, a CMV having the sequence 5'-agucuggaugGCTAAgccgcccuca-3' (SEQ ID No. 26) is considered to have the sequence of SEQ ID No: 4, wherein the lower case letters denote ribo-type nucleobases and the UPPER CASE letters denote deoxyribo-type nucleobases.

Subjects can be treated with a recombinagenic oligonucleobase specific for Apo B or Apo E according to the guidance of the Factor IX example below. More particularly the recombinagenic oligonucleobase can be given in divided doses at intervals that permit determining of the phenotypic effect of the dose, i.e., evaluation of the extent of the decline in LDL cholesterol and observation for adverse reactions. A reduction of the subject's fasting LDL serum cholesterol to below the level of the 5th percentile of the age-matched population (80-90 mg/dl) can be used as a therapeutic end point; alternatively reduction of fasting LDL serum cholesterol to below the average age-matched normal value (100-140) can be used. The number and size of the dose(s) can be modified to control the extent of the phenotypic effects. In the event that reversal of the specific genetic changes appear desirable, a recombinagenic oligonucleobase having a sequence appropriate to reverse the specific changes can be administered so that the fraction of unmodified Apo B or Apo E genes can be increased. Modification of the dose size and number and the administration of a reversing recombinagenic oligonucleobase permits the adjustment of the number of altered genes in the subject so that a predetermined amount of the phenotypic change can be effected.

6.2.1 Specific Alterations of the Apo B Gene

SEQ ID No. 1 contains the Apo B amino acid sequence and SEQ ID No. 2 contains the Apo B cDNA sequence.

The level of serum cholesterol and particularly of LDL-associated cholesterol can be reduced in a subject by introducing mutations into the subject's hepatic Apo B genes. The mutation can be any mutation that causes termination of the Apo B translation product between amino acid 1433 (Apo B 31) and amino acid 3974 (Apo B 87). (The amino acid numbering for Apo B in this specification refers to the 4553 amino acid primary translation product, i.e., mature Apo B100 plus the 27 amino acid leader sequence. Mature Apo B 100 consists of 4536 amino acids and mature Apo B 48 consists

of 2152 amino acids.) Preferably the translation product is terminated between amino acids 1841 (Apo B 40) and 2975 (Apo 65). The translation product can be terminated by introducing a frameshift mutation, i.e., by adding or deleting one or two nucleotides from the gene, or by introducing a stop codon (a TAA, TAG or TGA). The preferred stop codon is TAA. To monitor the introduction of the mutation it is preferred to have the mutation introduce or remove a palindromic sequence, which is the substrate of a restriction enzyme.

The sequence of the CMV is selected to have two homologous regions of at least 10 nucleobases and preferably at least 12 nucleobases each with a fragment of the Apo B gene located between nucleotides encoding amino acid 1433 (nt 4425) and 3974 (nt 12,048) and preferably located between the nucleotides encoding amino acids 1841 (nt 5649) and 2975 (nt 9051). In this specification, nt 6666 is the first nucleotide of codon 2180, the nucleotide that is converted by APOBE. In a preferred embodiment, the two homologous regions are separated by a single nucleobase in the sequence of the Apo B gene, where the CMV introduces a base substitution in the Apo B gene. Alternatively, the two homology regions can be adjacent in the Apo B gene and separated by a single or double nucleobase in the CMV, such that a one or two base insertion results from the action of the CMV on the Apo B gene. Alternatively, the homologous regions can be separated in the Apo B gene by one or two nucleotides that are deleted from the sequence of the CMV, such that the action of the CMV results in a one or two base deletion in the gene.

Nucleotides 4425-12,048 of the Apo B cDNA are encoded by exon 26 (nt 4342-11913), exon 27 (nt 11914 - 12028) and exon 28 (nt 12029-12212); see Table I, and GENBANK Accession No. 19828, which is hereby incorporated by reference. When an alteration is to be made at a position 3' of nt 11913, attention must be paid to the exon/intron boundary. Mutations that are located within 10-15 nucleotides of the exon/intron boundary must be identified so that the homology region of the CMV continues with the sequence of the intron and not the exon.

The homologous regions can be each from 10 to about 15 nucleobases in length; the two regions need not be of the same length. The fraction of nucleobases that contain a guanine or cytosine base is a design consideration (the GC fraction). It is preferred that

when the homologous region contains 12 or fewer nucleobases, the GC fraction be at least 33% and preferably at least 50%. When the GC fraction is less than 33% the length of the homologous regions is preferably 13, 14 or 15 nucleobases.

Table I contains 18 exemplary embodiments, SEQ ID No. 4-21 and Table III contains 9 exemplary embodiments, SEQ ID No. 22-25 and 54-58, of CMV sufficient for the practice of the embodiments of the invention described in this section. Suitable CMV can be made using nt 3-23 of SEQ ID No. 4-10, 12, and 16-20. SEQ ID NO. 11 and 13-15 have a lower GC fraction; CMV sufficient for the practice of the invention can be made containing residues 3-25 of SEQ ID NO. 11 and 13-15.

6.2.2 Specific Alterations of the Apo E Gene

In a further embodiment, the invention consists of introducing specific alterations to the Apo E gene. E4 homozygous individuals are at increased risk for atherosclerosis, particularly coronary artery disease, and Alzheimer's disease. Therefore, one embodiment of the present invention is the introduction of the substitution Arg→Cys at residues 112, to convert an E4 allele to an E3 allele, and optionally at residue 158 to convert an E3 or E4 allele into an E2 allele of an Apo E gene of an hepatocyte of a subject. The substitutions can be introduced using an oligonucleobase containing the sequence of nt 3-23 of SEQ ID No. 22 and No. 23 or complement thereof and more preferably of an oligonucleobase containing SEQ ID No. 22 and No. 23 or complement thereof. In addition, in individuals lacking genetic or environment stressors, the E2 allele results in a lowered LDL level and a decreased risk of atherosclerosis and coronary artery disease. Thus, these risks in an E3/E3 individual can be reduced by introduction of the $(Arg\rightarrow Cys)^{158}$ substitution to convert the individual Apo E genes to E2 alleles.

Apo E2/E2 homozygous individuals who are suffering from Type III hyperlipidemia can be treated by converting E2 alleles to E3 alleles by making a $Cys\rightarrow Arg^{158}$ substitution. Such a substitution can be made using an oligonucleobase containing the sequence of nt 3-23 of SEQ ID No. 24 or complement thereof and more preferably of an oligonucleobase containing SEQ ID No. 24 or complement thereof.

Independent of the Apo E allele, individuals who are homozygous for -491-A are at increased risk to develop Alzheimer's Disease. Bullido, M.J., 1998, et al., Nature

Genetics **18,** 69-71. These individuals can be advantageously treated with an oligonucleobase containing the sequence of nt 3-23 of SEQ ID No. 25.

6.2.3 Repair of Mutations of the Apo B and Apo E Gene

SEQ ID No. 3 contains the Apo E genomic DNA sequence.

A further embodiment of the invention concerns the use of CMV to repair mutations in the Apo B and Apo E genes that cause hypobetalipoproteinemia and dysbetaliproteinemia, respectively. Mutations that are located within 10-15 nucleotides of the exon/intron boundary must be identified so that the homology region of the CMV continues with the sequence of the intron and not the exon. The genomic sequence of Apo E4 indicating the exon and intron boundaries is given in Paik et al., 1985, Proc. Natl. Acad. Sci. **82,** 3445, which is hereby incorporated by reference. The exon/intron boundaries of the Apo B gene are given in Table II along with the GENBANK accession numbers for the genomic sequence of Apo B.

6.2.4 Specific Alterations of the Apo A1 Gene

Apo A1 is a 243 amino acid protein. Amino acids 99-230 are encoded by six tandem duplications of a 66 base pair prototype sequence. The duplications are between 80 % and 64 % homologous to the consensus sequence. Without limitation as to theory the conformation of amino acids 120 to 230 is believed to be helical. The sequence of the amino acids is such that the helix is amphipathic, i.e., the helix has a hydrophobic face and a hydrophilic face, which contains polar amino acids.

The mutations most suitable for the practice of the invention are substitutions of polar amino acids by cysteine. Particularly suitable mutations are at arginine residues that are located next to other polar amino acids because the arginine codon used in apo A1 (CGC) can be converted into a TGC cysteine codon by a single base change. Thus, particularly suitable sites for mutations according to the present invention are arginine 149, 151, 153, 171 and 173.

6.3 FORMULATIONS SUITABLE FOR *IN VIVO* USE

The prior art formulations of CMV and a macromolecular carrier are of limited utility for *in vivo* use because of their low capacity for CMV and because the CMV is not protected from extracellular enzymes. The invention provides three alternative macromolecular carriers that overcome the limitations of the prior art. The carriers are polyethylenimine (PEI), aqueous-cored lipid vesicles, which are also termed unilamellar liposomes and lipid nanospheres.

Each of the carriers can be further provided with a ligand that is complementary to a cell-surface protein of the target cell. Such ligands are useful to increase both the amount and specificity of the uptake of CMV into the targeted cell. In one embodiment of the invention the target cell is a hepatocyte and the ligand is a galactose saccharide or lactose disaccharide that binds to the asialoglycoprotein receptor.

6.3.1 Polycationic Carriers

The invention can be practiced using any polycation that is non-toxic when administered to cells *in vitro* or to subjects *in vivo*. Suitable examples include polybasic amino acids such as polylysine, polyarginine, basic proteins such as histone H1, and synthetic polymers such as the branched-chain polyethylenimine:

$$(\text{-NHCH}_2\text{CH}_2\text{-})_X\,[\text{-N(CH}_2\text{CH}_2\text{NH}_2)\text{CH}_2\text{CH}_2\text{-}]_Y.$$

The invention can be practiced with any branched chain polyethylenimine (PEI) having an average molecular weight of greater than about 500 daltons, preferably greater than between about 10 Kd and more preferably about 25 Kd (mass average molecular weight determined by light scattering) The upper limit of suitability is determined by the toxicity and solubility of the PEI. Toxicity and insolubility of molecular weights greater than about 1.3 Md makes such PEI material less suitable. The use of high molecular weight PEI as a carrier to transfect a cell with DNA is described in Boussif, O. et al., 1995, Proc. Natl. Acad. Sci. **92**, 7297, which is hereby incorporated by reference. PEI solutions can be prepared according to the procedure of Boussif et al.

The CMV carrier complex is formed by mixing an aqueous solution of CMV and a neutral aqueous solution of PEI at a ratio of between 9 and 4 PEI nitrogens per CMV phosphate. In a preferred embodiment the ratio is 6. The complex can be formed, for

example, by mixing a 10 mM solution of PEI, at pH 7.0 in 0.15 M NaCl with CMV to form a final CMV concentration of between 100 and 500 nM.

In addition a ligand for a clathrin-coated pit receptor can be attached to the polycation or to a fraction of the polycations. In one embodiment the ligand is a saccharide or disaccharide that binds to the asialoglycoprotein receptor, such as lactose, galactose, or N-acetylgalactosamine. Any technique can be used to attach the ligands. The optimal ratio of ligand to polyethylene subunit can be determined by fluorescently labeling the CMV and injecting fluorescent CMV/molecular carrier/ligand complexes directly into the tissue of interest and determining the extent of fluorescent uptake according to the method of Kren et al., 1997, Hepatology **25**, 1462-1468.

Good results can be obtained using a 1:1 mixture of lactosylated PEI having a ratio of 0.4-0.8 lactosyl moieties per nitrogen and unmodified PEI. The mixture is used in a ratio of between 4 and 9 PEI nitrogens per CMV phosphate. A preferred ratio of oligonucleotide phosphate to nitrogen is 1:6. Good results can be obtained with PEIs having a mass average molecular weight of 25 Kd and 800 Kd which are commercially available from Aldrich Chemical Co., Catalog No. 40,872-7 and 18,197-8, respectively. Linear PEI such as that described in Ferrarri, S., et al., 1997, Gene Therapy **4**, 1100-1106 and sold under the trademark EXGEN 500™ is particularly suitable for the practice of the invention because of its lower toxicity compared to branched-chain PEI.

In an alternative embodiment the polycationic carrier can be a basic protein such as histone H1, which can be substituted with a ligand for a clathrin-coated pit receptor. A 1:1 (w/w) mixture of histone and CMV can be used to practice the invention.

6.3.2 Lipids that Are Useful in Carriers

The selection of lipids for incorporation into the lipid vesicle and lipid nanosphere carriers of the invention is not critical. Lipid nanospheres can be constructed using semi-purified lipid biological preparations, e.g., soybean oil (Sigma Chem. Co.) and egg phosphatidyl choline (EPC) (Avanti Polar Lipids). Other lipids that are useful in the preparation of lipid nanospheres and/or lipid vesicles include neutral lipids, e.g., dioleoyl phosphatidylcholine (DOPC), and dioleoyl phosphatidyl ethanolamine (DOPE), anionic lipids, e.g., dioleoyl phosphatidyl serine (DOPS) and cationic lipids, e.g., dioleoyl

trimethyl ammonium propane (DOTAP), dioctadecyldiamidoglycyl spermine (DOGS), dioleoyl trimethyl ammonium (DOTMA) and DOSPER (1,3-di-oleoyloxy-2-(6-carboxy-spermyl)-propyl-amide tetraacetate, commercially available from Boehringer-Mannheim). Additional examples of lipids that can be used in the invention can be found in Gao, X. and Huany, L., 1995, Gene Therapy **2**, 710. Saccharide ligands can be added in the form of saccharide cerebrosides, e.g., lactosylcerebroside or galactocerebroside (Avanti Polar Lipids).

The particular choice of lipid is not critical. Hydrogenated EPC or lysolecithin can be used in place of EPC. DPPC (dipalmitoyl phosphatidylcholine), can be incorporated to improve the efficacy and/or stability of the delivery system.

6.3.3 The Construction of Lipid Nanosphere Carriers

Lipid nanospheres can be constructed by the following process. A methanol or chloroform methanol solution of phospholipids is added to a small test tube and the solvent removed by a nitrogen stream to leave a lipid film. A lipophilic salt of CMV is formed by mixing an aqueous saline solution of CMV with an ethanolic solution of a cationic lipid. Good results can be obtained when the cationic species are in about a 4 fold molar excess relative to the CMV anions (phosphates). The lipophilic CMV salt solution is added to the lipid film, vortexed gently followed by the addition of an amount of neutral lipid equal in weight to the phospholipids. The concentration of CMV can be up to about 3% (w/w) of the total amount of lipid.

After addition of the neutral lipid, the emulsion is sonicated at 4°C for about 1 hour until the formation of a milky suspension with no obvious signs of separation. The suspension is extruded through polycarbonate filters until a final diameter of about 50 nm is achieved. When the target cell is a reticuloendothelial cell the preferred diameter of the lipid nanospheres is about 100-200 nm. The CMV-carrying lipid nanospheres can then be washed and placed into a pharmaceutically acceptable carrier or tissue culture medium. The capacity of lipid nanospheres is about 2.5 mg CMV/ 500 μl of a nanosphere suspension.

6.3.4 The Construction of Lipid Vesicles

A lipid film is formed by placing a chloroform methanol solution of lipid in a tube and removing the solvent by a nitrogen stream. An aqueous saline solution of CMV is added such that the amount of CMV is between 20% and 50% (w/w) of the amount of lipid, and the amount of aqueous solvent is about 80% (w/w) of the amount of lipid in the final mixture. After gentle vortexing the liposome-containing liquid is forced through successively finer polycarbonate filter membranes until a final diameter of about 50 nm is achieved. The passage through the successively finer polycarbonate filter results in the conversion of polylamellar liposomes into unilamellar liposomes, i.e., vesicles. When the target cell is a reticuloendothelial cell the preferred diameter of the lipid nanospheres is about 100-200 nm. The CMV-carrying lipid nanospheres can then be washed and placed into a pharmaceutically acceptable carrier or tissue culture medium.

The CMV are entrapped in the aqueous core of the vesicles. About 50% of the added CMV is entrapped.

A variation of the basic procedure comprises the formation of an aqueous solution containing a PEI/CMV condensate at a ratio of about 4 PEI imines per CMV phosphate. The condensate can be particularly useful when the liposomes are positively charged, i.e., the lipid vesicle contains a concentration of cations of cationic lipids such as DOTAP, DOTMA or DOSPER, greater than the concentration of anions of anionic lipids such as DOPS. The capacity of lipid vesicles is about 150 μg CMV per 500 μl of a lipid vesicle suspension.

In a preferred embodiment the lipid vesicles contain a mixture of the anionic phospholipid, DOPS, and a neutral lipid such as DOPE or DOPC. Other negatively charged phospholipids that can be used to make lipid vesicles include dioleoyl phosphatidic acid (DOPA) and dioleoyl phosphatidyl glycerol (DOPG). In a more preferred embodiment the neutral lipid is DOPC and the ratio of DOPS:DOPC is between 2:1 and 1:2 and is preferably about 1:1. The ratio of negatively charged to neutral lipid should be greater than 1:9 because the presence of less than 10% charged lipid results in instability of the lipid vesicles because of vesicle fusion.

A particular lipid vesicle formulation can be tested by using the formulation to transfect a target cell population with a plasmid of about 5.0 kb in length that expresses

some readily detectable product in the transfected target cell. Lipid vesicles can be used to transfect a cell with the plasmid if the plasmid is condensed with PEI at an imine:phosphate ratio of about 9-4:1. The capacity of the lipid vesicle formulation to transfect a cell with a plasmid is indicative of the formulation's capacity to introduce a CMV into a cell and effect a transmutation.

Certain lipids, particularly the polycationic lipids, can be toxic to certain cell lines and primary cell cultures. The formulation of the lipid vesicles should be adjusted to avoid such toxic lipids.

Ligands for clathrin-coated pit receptors can be introduced into the lipid vesicles by a variety of means. Cerebrosides, such as lactocerebroside or galactocerebroside can be introduced into the lipid mixture and are incorporated into the vesicle to produce a ligand for the asialoglycoprotein receptor.

In an alternative embodiment the lipid vesicle further comprises an integral membrane protein that inserts itself into the lipid bilayer of the vesicle. In a specific embodiment the protein is a fusigenic (F-protein) from the virus alternatively termed Sendai Virus or Hemagglutinating Virus of Japan (HVJ). The preparation and use of F-protein containing lipid vesicles to introduce DNA into liver, myocardial and endothelial cells have been reported. See, e.g., U.S. Patent No. 5,683,866, International Application PCT JP97/ 00612 (published as WO 97/31656). See also, Ramani, K., et al., 1996, FEBS Letters **404**, 164-168; Kaneda, Y., et al., 1989, J. Biol. Chem. **264**, 121126-12129; Kaneda, Y., et al., 1989, Science **243**, 375; Dzau, V.J., et al., Proc. Natl. Acad. Sci. **93**, 11421-11425; Aoki, M., et al., 1997, J.Mol.Cardiol. **29**, 949-959.

6.4 The Use of the Formulations *In Vivo*

The CMV of the invention can be parenterally administered directly to the target organ at a dose of between 50 and 250 μg/gm. When the target organ is the liver muscle or kidney, the CMV/macromolecular carrier complex can be injected directly into the organ. When the target organ is the liver, intravenous injection into the hepatic or portal veins of a liver, having temporarily obstructed circulation can be used. Alternatively the CMV/macromolecular complex can further comprise a hepatic targeting ligand, such as a

lactosyl or galactosyl saccharide, which allows for administration of the CMV/macromolecular complex intravenously into the general circulation.

When the target organ is the lung or a tissue thereof, e.g., the bronchiolar epithelium CMV/macromolecular complex can be administered by aerosol. Small particle aerosol delivery of liposomal/DNA complexes is described in Schwarz L.A., et al., 1996, Human Gene Therapy 7, 731-741.

When the target organ is the vascular endothelium, as for example in von Willebrand's Disease, the CMV/macromolecular complex can be delivered directly into the systemic circulation. Other organs can be targeted by use of liposomes that are provided with ligands that enable the liposome to be extravasated through the endothelial cells of the circulatory system.

For enzymatic defects, therapeutic effects can be obtained by correcting the genes of about 1% of the cells of the affected tissue. In a tissue in which the parenchymal cells have an extended life, such as the liver, treatments with CMV can be repeatedly performed to obtain an increased therapeutic effect.

7. EXAMPLES

7.1 CMV/MACROMOLECULAR CARRIER COMPLEXES

7.1.1 Lipid Nanospheres

Materials

Egg phosphatidylcholine (EPC), DOTAP and galactocerebroside (Gc) (Avanti Polar Lipids); soybean oil (Sigma Chemical Co.); dioctadecyldiamidoglycyl spermine (DOGS®) (Promega).

Methods

EPC, DOTAP and Gc were previously dissolved at defined concentrations in chloroform or anhydrous methanol and stored in small glass vials in desiccated containers at -20°C until use. EPC (40-45 mg), DOTAP (200 μg) and Gc (43 μg) solutions were aliquoted into a small 10 x 75 mm borosilicate tube and solvents removed under a stream of nitrogen. CMV were diluted in 0.15 M NaCl (~80-125 μg/250-300μl); DOGS (as a 10 mg/ml solution in ethanol) was diluted into 250-300 μl 0.15 M NaCl at 3-5 times the weight of added CMV. The two solutions were mildly vortexed to mix contents and then CMV solution was added slowly to the DOGS solution. The contents were mixed by gentle tapping and inverting the tube a few times. The DOGS-complex solution was added to the dried lipids followed by soybean oil (40-45 mg), the mixture was vortexed on high for a few seconds and bath sonicated in a FS-15 (Fisher Scientific) bath sonicator for ~1 hr in a 4°C temperature controlled room. Occasionally, the tube was removed from the bath and vortexed. When a uniform looking, milky suspension was formed (with no obvious separation of oil droplets), it was extruded through a series of polycarbonate membranes down to a pore size of 50 nm. Preparations were stored at 4°C until use and vortexed before use.

7.1.2 Negatively Charged Targeted Lipid Vesicles

Materials

Dioleoyl phosphatidylcholine (DOPC), dioleoyl phosphatidylserine (DOPS), galactocerebroside (Gc) or lactosylcerebroside, (Avanti Polar Lipids).

Methods

DOPS, DOPC and Gc at a molar ratio of 1:1:0.16 (500 μg total lipid) were dissolved in chloroform:methanol (1:1 v/v) and then dried under a stream of nitrogen to obtain a uniform lipid film. The CMV were diluted in 500 μl of 0.15 M NaCl (approximately 100-250 μg/500μl). The solution was added to the lipid film at room temperature. Lipids were dispersed entirely by alternate mild vortexing and warming (in a water bath at 37-42°C). After a uniform milky suspension was formed, it was extruded through a series of polycarbonate membranes (pore sizes 0.8, 0.4, 0.2, 0.1 and 0.05μm) using a Liposofast® mini-extruder. Extrusion was done 5 to 7 times through each pore size. After preparation, lipid vesicles were stored at 4°C until use. Under these conditions the lipid vesicles were stable for at least one month. The final product can be lyophilized.

7.1.3 Neutral Targeted Lipid Vesicles

Materials

Dioleoyl phosphatidylcholine (DOPC), dioleoyl phosphatidylethanolamine (DOPE), galactocerebroside (Gc) or lactosylcerebroside, (Avanti Polar Lipids).

Methods

DOPC, DOPE and Gc (1:1:0.16 molar ratio) or DOPC:Gc (1:0.08) were dissolved in chloroform:methanol (1:1 v/v) and then dried under a stream of nitrogen to obtain a uniform lipid film. The oligonucleotides (or chimeric molecules) were diluted in 500 μl of 0.15 M NaCl (approximately 100-250 μg/500 μl). The solution was added to the lipid film at room temperature. Lipids were dispersed entirely by alternate mild vortexing and warming (in a water bath at 37-42°C). After a uniform milky suspension was formed, it was extruded through a series of polycarbonate membranes (pore sizes 0.8, 0.4, 0.2, 0.1 and 0.05 μm) using a Liposofast® mini-extruder. Extrusion was done 5 to 7 times through each pore size. After preparation, lipid vesicles were stored at 4°C until use. The size of the lipid vesicles of the preparation was stable for about 5 days.

7.1.4 Positively Charged Targeted Lipid Vesicles

Materials

Dioleoyl phosphatidylcholine (DOPC), dioleoyl trimethylammonium propane (DOTAP), galactocerebroside (Gc) or lactosylcerebroside, (Avanti Polar Lipids). Polyethylenimine (PEI) (M.W. 800 Kd), Fluka Chemicals.

Methods

DOPC, DOTAP and Gc (6:1:0.56 molar ratio) (500 μg total lipid) were dissolved in chloroform:methanol (1:1 v/v) and then dried under a stream of nitrogen to obtain a uniform lipid film. PEI was diluted to a concentration of 45 mg/100 ml using water. pH of the solution was adjusted to ~7.6 using HCl. This PEI stock solution was prepared fresh each time and was equivalent to approximately 50 nmol amine/μl. CMV were diluted into 0.15 M NaCl at a concentration of ~125 μg in 250 μl. PEI was further diluted into 250 μl 0.15 M NaCl so that approximately 4 moles of PEI amine were present per mole of oligonucleotide/chimeric phosphate. PEI solution was added drop-wise to the CMV solution (both at room temperature) and vortexed for 5-10 minutes. The PEI-complex solution was then added to the lipid film and the lipids dispersed as described above. After a uniform milky suspension was formed, it was extruded through a series of polycarbonate membranes (pore sizes 0.8, 0.4, 0.2, 0.1 and 0.05 μm) using a Liposofast® mini-extruder. Extrusion was done 5 to 7 times through each pore size. After preparation lipid vesicles were stored at 4°C until use. Under these conditions the lipid vesicles were stable for at least one month. For longer and improved stability the final product can be lyophilized.

7.1.5 Lactosylated-PEI/PEI Complexes

PEI (25 kDa) was purchased from Aldrich Chemical (Milwaukee, WI). PEI (800 kDa) was purchased from Fluka chemicals (Ronkonkoma, NY, USA) . Lactosylation of the PEI was carried out by modification of a previously described method for the conjugation of oligosaccharides to proteins. Briefly, 3 to 5 ml of PEI (0.1 to 1.2 $M_{monomer}$) in ammonium acetate (0.2 M) or Tris buffer (0.2 M) (pH 7.6) solution was incubated with 7 to 8 mg of sodium cyanoborohydride (Sigma Chemical Co., St. Louis, MO) and

approximately 30 mg of lactose monohydrate (Sigma Chemical Co., St. Louis, MO). Reaction was carried out in polypropylene tubes, tightly capped in a 37°C shaking water bath. After 10 days the reaction mixture was dialyzed against distilled water (500 ml) for 48 h with 1 to 2 changes of water. The purified complex was sterile filtered through 0.2 μm filter and stored at 4°C. The amount of sugar (as galactose) associated with PEI was determined by the phenol-sulphuric acid method.

The number of moles of free amine (primary + secondary) in the lactosylated PEI was determined as follows: a standard curve was set up using a 0.02M stock solution of PEI; several aliquots of the stock were diluted to 1ml using deionized water in glass tubes, then 50 μl of Ninhydrin reagent (Sigma Chemical Co., St. Louis, Mo) was added to each tube and vortexed vigorously for 10 sec. Color development was allowed to proceed at room temperature for 10 to 12 min. and then O.D. was read (within 4 minutes) at 485 nm on a Beckman DU-64 spectrophotometer. 20 to 50 μl aliquots of the L-PEI samples were treated as above and the number of moles of free amine was determined from the standard curve. Lactosylated-PEI (L-PEI) complexes were prepared as follows: an equivalent of 3 mmol of amine as L-PEI and 3 mmol of amine as PEI, per mmol of RNA/DNA phosphate, were mixed together and diluted in 0.15M NaCl as required; the mixture was added dropwise to a solution of the chimeric and vortexed for 5 min.

To verify complete association of the chimeric oligonucleotides with PEI or L-PEI, gel analysis (4% LMP agarose) of the uncomplexed and complexed chimerics was performed. To determine the degree of protection against nuclease degradation provided by complexation of the chimerics, samples were treated with RNAse and DNAse. After a chloroform phenol extraction, the complexes were dissociated using heparin (50 units/μg nucleic acid) and the products analyzed on a 4% LMP agarose gel.

7.2 Demonstration of PEI/CMV Mediated Alteration of Rat and Human Factor IX

Materials. Fetal bovine serum was obtained from Atlanta Biologicals, Inc. (Atlanta, GA). The terminal transferase, fluorescein-12-dUTP, Expand™ high fidelity PCR system, dNTPs and high pure PCR template preparation kit were obtained from Boehringer Mannheim Corp. (Indianapolis, IN). Reflection™ NEF-496 autoradiography

film and Reflection™ NEF-491 intensifying screens were from DuPont NEN® Research Products (Boston, MA). Polyethylenimine (PEI) 800 kDa was obtained from Fluka Chemical Corp. (Ronkonkoma, NY). The [γ-^{32}P]ATP was obtained from ICN Biochemicals, Inc. (Costa Mesa, CA). pCR™2.1 was obtained from Invitrogen (San Diego, CA). OPTIMEM™, Dulbecco's modified Eagle's medium, William's E medium and oligonucleotides 365-A and 365-C were from Life Technologies, Inc. (Gaithersburg, MD). Spin filters of 30,000 mol wt cutoff were purchased from Millipore Corp. (Bedford, MA). DiI and SlowFade™ antifade mounting medium were obtained from Molecular Probes, Inc. (Eugene, OR). T4 polynucleotide kinase was purchased from New England Biolabs, Inc. (Beverly, MA). MSI MagnaGraph membrane was purchased from Micron Separations, Inc. (Westboro, MA). The primers used for PCR amplification were obtained from Oligos Etc., Inc. (Wilsonville, OR). Tetramethylammonium chloride was purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals were molecular biology or reagent grade and purchased from Aldrich Chemical Company (Milwaukee, WI), Curtin Matheson Scientific, Inc. (Eden Prairie, MN), and Fisher Scientific (Itasca, IL).

Oligonucleotide synthesis. Chimeric RNA/DNA oligonucleotides HIXF, RIXF and RIXR were synthesized. The CMV were prepared with DNA and 2'-O-methyl RNA phosphoramidite nucleoside monomers on an ABI 394 synthesizer. The DNA phosphoramidite exocyclic amine groups were protected with benzoyl (adenosine and cytidine) and isobutyryl (guanosine). The protective groups on the 2'-O-methyl RNA phosphoramidites were phenoxyacetyl for adenosine, isobutyryl for cytidine, and dimethylformamide for guanosine. The base protecting groups were removed following synthesis by heating in ethanol/concentrated ammonium hydroxide for 20 h at 55°C. The crude oligonucleotides were electrophoresed on 15% polyacrylamide gels containing 7 M urea, and the DNA visualized using UV shadowing. The chimeric molecules were eluted from the gel slices, concentrated by precipitation and desalted using G-25 spin columns. Greater than 95% of the purified oligonucleotides were full length.

The sequence of the wild type and "mutant" rat Factor IX are

(SEQ ID No. 27) 365
wt AAA GAT TCA TGT GAA GGA GAT AGT GGG GGA CCC CAT GTT
Lys Asp Ser Cys Glu Gly Asp Ser Gly Gly Pro His Val
(SEQ ID No. 28)

(SEQ ID No. 29)
mt AAA GAT TCA TGT GAA GGA GAT **C**GT GGG GGA CCC CAT GTT
Arg

The structure of the RIXR, RIXF and HIXR CMV is as follows:

Chimeric Oligonucleotides

RIXR (SEQ ID No. 30)

TGCGCG-ccccaggggg**TG**<u>**C**</u>**TA**gaggaaguguT
T T
T T
TCGCGC GGGGTCCCCC**AC**<u>**G**</u>**AT**CTCCTTCACAT
3' 5'

$RIXR_C$ (SEQ ID No. 31)

TGCGCG-acacuuccuc**TA**<u>**G**</u>**CA**cccccuggggT
T T
T T
TCGCGC TGTGAAGGAG**AT**<u>**C**</u>**GT**GGGGGACCCCT
3' 5'

RIXF (SEQ ID No. 32)

TGCGCG-acacuuccuc**TA**<u>**G**</u>**CA**cccccuggggT
T T
T T
TCGCGC TGTGAAGGAG**AT**<u>**C**</u>**GT**GGGGGACCCCT
3' 5'

```
  HIXF                                (SEQ ID No. 33)
 TGCGCG-acaguuccucTAGCAcccccuggggT
T                                                  T
T                                                  T
 TCGCGC TGTCAAGGAGATCGTGGGGGACCCCT
       3' 5'
```

Uppercase letters are deoxyribonucleotides, lower case letters are 2'OMe-ribonucleotides. The nucleotide of the heterologous region is underlined.

Cell Culture, transfections and hepatocyte isolation. HuH-7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% (vol/vol) heat inactivated fetal bovine serum in a humidified CO_2 atmosphere at 37°C. Twenty four hours prior to transfection 1 x 10^5 cells were plated per 35 mm culture dish. At the time of transfection, the cells were rinsed twice with OPTIMEM™ media and transfections were performed in 1 ml of the same media. Eighteen hours after transfection, 2 ml of Dulbecco's modified Eagle's medium containing 20% (vol/vol) heat inactivated fetal bovine serum was added to each 35 mm dish and the cells maintained for an additional 30 h prior to harvesting for DNA isolation. A PEI (800 kDa) 10 mM stock solution, pH 7.0, was prepared. Briefly, the chimeric oligonucleotides were transfected with 10 mM PEI at 9 equivalents of PEI nitrogen per chimeric phosphate in 100 μl of 0.15 M NaCl at final concentrations of either 150 nM (4 μg), 300 nM (8 μg) and 450 nM (12 μg). After 18 h, an additional 2 ml of medium was added and reduced the chimeric concentrations to 50 nM, 100 nM, and 150 nM, respectively, for the remaining 30 h of culture. HuH-7 vehicle control transfections utilized the same amount of PEI as was used in the HuIXF transfections, but substituted an equal volume of 10 mM Tris-HCl pH 7.6 for the oligonucleotides.

Primary rat hepatocytes were isolated from 250 g male Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN) by a two step collagenase perfusion as previously described (Fan et al., Oncogene 12:1909-1919, 1996, which is hereby incorporated by reference) and plated on Primaria™ plates at a density of 4 x 10^5 cells per 35 mm dish. The cultures were maintained in William's E medium supplemented with 10% heat inactivated FBS, 26 mM sodium bicarbonate, 23 mM HEPES, 0.01 U/ml

insulin, 2 mM L-glutamine, 10 nM dexamethasone, 5.5 mM glucose, 100 U/ml penicillin and 100 U/ml streptomycin. Twenty four hours after plating, the hepatocytes were washed twice with the same medium and 1 ml of fresh medium added and the cells transfected using PEI/chimeric oligonucleotide complexes at the identical concentrations as for the HuH-7 cells. After 18 h, an additional 2 ml of the medium was added and the cells harvested 6 or 30 h later.

Direct injection of chimeric oligonucleotides into liver. Male Sprague-Dawley rats (~175 g) were maintained on a standard 12 h light-dark cycle and fed *ad libitum* standard laboratory chow. The rats were anesthetized, a midline incision made the liver exposed. A clamp was placed on the hepatic and portal veins as they enter the caudate lobe, and 75 μg of the 1:9 chimeric/PEI complex was injected in a final volume of 250 - 300 μl directly into the caudate lobe. The lobe remained ligated for 15 min and then blood flow was restored by removing the clamp. After suturing the incision the animals were allowed to recover from the anesthesia and given food and water *ad libitum*. Vehicle controls were done substituting an equal volume of Tris-HCl pH 7.6 for the chimeric oligonucleotides. Twenty-four and 48 h post-injection the animals were sacrificed, the caudate lobe removed and the tissue around the injection site dissected for DNA isolation. DNA was isolated and the terminal exon of the rat factor IX gene was amplified by PCR.

Nuclear uptake of the chimeric molecules. Chimeric duplexes were 3' end-labeled using terminal transferase and fluorescein-12-dUTP according to the manufacturer's recommendation, and were then mixed with unlabeled oligonucleotides at a 2:3 ratio. Transfections were performed as described above and after 24 h the cells were fixed in phosphate buffered saline, pH 7.4, containing 4% paraformaldehyde (wt/vol) for 10 min at room temperature. Following fixation, the cells were counterstained using a 5 μM solution of DiI in 0.32 M sucrose for 10 min according to the manufacturer's recommendation. After washing with 0.32 M sucrose and then phosphate buffered saline, pH 7.4, the cells were coversliped using SlowFade™ antifade mounting medium in phosphate buffered saline and examined using a MRC1000 confocal microscope (BioRad, Inc., Hercules, CA). The caudate lobes of liver *in situ* were injected with fluorescently-labeled chimerics as described above and harvested 24 h post-

injection. The lobes were bisected longitudinally, embedded using OCT and frozen. Cryosections were cut ~10 μm thick, fixed for 10 min at room temperature using phosphate buffered saline, pH 7.4, containing 4% paraformaldehyde (wt/vol). Following fixation, the cells were counterstained using a 5 μM solution of DiI in 0.32 M sucrose for 10 min according to the manufacturer's recommendation. After washing with 0.32 M sucrose and then phosphate buffered saline, pH 7.4, the sections were coversliped using SlowFade™ antifade mounting medium and examined using a MRC1000 confocal microscope (BioRad, Inc.). The collection series for the fixed cells and sectioned tissue were made at 1 μm steps to establish the presence of the chimeric in the nucleus.

DNA isolation and cloning. The cells were harvested by scrapping 24 and 48 h after transfection. Genomic DNA larger than 100-150 base pairs was isolated using the high pure PCR template preparation kit according to the manufacturer's recommendation. PCR amplification of a 317-nt fragment of the eighth exon in the human liver factor IX gene was performed with 500 ng of the isolated DNA. The primers used were 5'-CATTGCTGACAAGGAATACACGAAC-3' (SEQ ID No. 34) and 5'-ATTTGCCTTTCATTGCACACTCTTC-3' (SEQ ID No. 35) corresponding to nucleotides 1008-1032 and 1300-1324, respectively, of the human factor IX cDNA. Primers were annealed at 58°C for 20 sec, elongation was for 45 sec at 72°C and denaturation proceeded for 45 sec at 94°C. The sample was amplified for 30 cycles using Expand Hi-fidelity™ polymerase. PCR amplification of a 374-nt fragment of the rat factor IX gene was performed with 500 ng of the isolated DNA from either the primary hepatocytes or liver caudate lobe. The primers used were 5'-ATTGCCTTGCTGGAACTGGATAAC-3' (SEQ ID No. 36) and 5'-TTGCCTTTCATTGCACATTCTTCAC-3' (SEQ ID No. 37) corresponding to nucleotides 433-457 and 782-806, respectively, of the rat factor IX cDNA. Primers were annealed at 59°C for 20 sec, elongation was for 45 sec at 72°C and denaturation proceeded for 45 sec at 94°C. The sample was amplified for 30 cycles using Expand Hi-fidelity™ polymerase. The PCR amplification products from both the human and rat factor IX genes were subcloned into the TA cloning vector pCR™2.1 according to the manufacturer's recommendations, and the ligated material used to transform frozen competent *Escherichia coli*.

Colony hybridization and sequencing. Eighteen to 20 h after plating, the colonies were lifted onto MSI MagnaGraph nylon filters, replicated and processed for hybridization according to the manufacturer's recommendation. The filters were hybridized for 24 h with 17 mer oligonucleotide probes 365-A (5'-AAGGAGATAGTGGGGGA-3') (SEQ ID No. 38) or 365-C (5'AAGGAGATCGTGGGGGA-3') (SEQ ID No. 39), where the underlined nucleotide is the target of the mutagenesis. The probes were ^{32}P-end-labeled using [γ-^{32}]ATP (>7,000 Ci/mmol) and T4 polynucleotide kinase according to the manufacturer's recommendations. Hybridizations were preformed at 37°C in 2X sodium chloride sodium citrate containing 1% SDS, 5X Denhardt's and 200 μg/ml denatured sonicated fish sperm DNA. After hybridization, the filters were rinsed in 1X sodium chloride phosphate EDTA, 0.5% SDS and then washed at 54°C for 1 h in 50 mM Tris-HCl, pH 8.0 containing 3 M tetramethylammonium chloride, 2 mM EDTA, pH 8.0, 0.1% SDS. Autoradiography was performed with NEN® Reflection film at -70°C using an intensifying screen. Plasmid DNA was prepared from colonies identified as hybridizing with 365-A or 365-C using Qiagen minprep kit (Chatsworth, CA) and subjected to automatic sequencing using the mp13 reverse primer on an ABI 370A sequencer (Perkin-Elmer, Corp., Foster City, CA).

Results *In Vivo*

Chimeric oligonucleotides were fluorescein-labeled and used to determine whether direct injection into the caudate lobe of the liver was feasible. The results indicated that the hepatocytes adjacent to the injection site within the caudate lobe showed uptake of the fluorescently-labeled chimeric molecules similar to that observed in isolated primary hepatocytes and HuH-7 cells. Although some punctate material was present in the cytoplasm, the labeled material was detected primarily in the nucleus. In fact, only nuclear labeling was observed in hepatocytes farthest from the injection site. The unlabeled PEI/RIXF chimeric complexes and vehicle controls were injected directly into the caudate lobe using the same protocol and the animals sacrificed 24 and 48 h post-injection. Liver DNA was isolated as described in Methods, subjected to PCR amplification of a 374 nt sequence spanning the targeted nt exchange site. Following

subcloning and transformation of *Escherichia coli* with the PCR amplified material, duplicate filter lifts of the transformed colonies were performed. The filters were hybridized with ^{32}-labeled 17-mer oligonucleotides specific for either 365-A (wild-type) or 365-C (factor IX mutation) and processed post-hybridization as described in Methods. Rats which received direct hepatic injection of the RIXF chimeric molecules exhibited a A→C conversion frequency of ~1% at both 24 and 48 h. In contrast, the vehicle controls showed no hybridization with the 365-C probe. Colonies that hybridized with the 365-C probe from the RIXF treated animals were cultured, the plasmid DNA isolated and subjected to sequencing to confirm the A→C conversion. The ends of the amplified 374-nt fragment correspond exactly with the primers and the only nucleotide change observed was an A→C at the targeted exchange site.

7.3 Demonstration of Lactosylated-PEI/CMV Mediated Alteration of Rat Factor IX

7.3.1 Results

CMV complexed to a mixture of lactosylated-PEI and PEI was prepared using the RIXR oligonucleotide as described in Section 6.1.5 above. A CMV directed to the complementary strand of the same region of the factor IX was also constructed ($RIXR_C$).

Conversion of the targeted nucleotide at Ser^{365} by the chimeric oligonucleotides

The nuclear localization of the fluorescently-labeled chimeric molecules indicated efficient transfection in the isolated rat hepatocytes. The cultured hepatocytes were then transfected with the unlabeled chimeric molecules factor $RIXR_C$ and RIXR at comparable concentrations using 800 kDa PEI as the carrier. Additionally, vehicle control transfections were performed simultaneously. Forty eight hours after transfection, the cells were harvested and the DNA isolated and processed for hybridization as described in Section 6.1.5. The A→C targeted nucleotide conversion at Ser^{365} was determined by hybridization of duplicate colony lifts of the PCR-amplified and cloned 374-nt stretch of exon 8 of the factor IX gene (Sarkar, B., Koeberl, D. D. & Somer, S. S., "Direct Sequencing of the activation peptide and the catalytic domain of the factor IX gene in six species," *Genomics*, 6, 133-143, 1990.) The 17 mer oligonucleotide probes used to distinguish between the wild-type 365-A (5'-AAGGAGATAGTGGGGGA-3') (SEQ ID No.

40) or converted 365-C (5'-AAGGAGATCGTGGGGGA-3') (SEQ ID No. 41) corresponded to nucleotides 710 through 726 of the cDNA sequence.

The overall frequency of conversion of the targeted nucleotide was calculated by dividing the number of clones hybridizing with the 365-C oligonucleotide by the total number of clones hybridizing with both oligonucleotide probes. The results are summarized in Table III for $RIXR_C$. A→C conversion at Ser^{365} was observed only in primary hepatocytes transfected with the RIXR or $RIXR_C$. Similar conversion frequencies were observed in hepatocytes transfected with RIXR or $RIXR_C$. Neither vehicle transfected cells nor those transfected with other chimeric oligonucleotides yielded any clones hybridizing with the 365-C oligonucleotide probe (unpublished observations). Additionally, no hybridization of the 365-C oligonucleotide probe was observed to clones derived from DNA isolated from untreated hepatocytes and PCR-amplified in the presence of 0.5 to 1.5 μg of the oligonucleotides. The A→C conversion rate in the isolated hepatocytes was also dose dependent using lactosylated PEI derivatives as described in Section 6.1.5 and was as high as 19%. RT-PCR and hybridization analysis of RNA isolated from cultured cells transfected in parallel with lactosylated PEIs demonstrated A→C conversion frequencies ranging from 11.9 to 22.3%.

Site-directed nucleotide exchange by chimeric oligonucleotides in intact liver

The fluorescein-labeled oligonucleotides were also used to determine cellular uptake of the chimeric molecules after direct injection into the caudate lobe of the liver. The results indicated that hepatocytes adjacent to the injection site in the caudate lobe showed uptake of the fluorescently-labeled chimerics similar to that observed in the isolated rat hepatocytes. Although some punctate material was present in the cytoplasm of the hepatocytes, the labeled material was primarily present in the nucleus. In fact, only nuclear labeling was observed in those areas farthest from the injection site. The unlabeled RIXR chimeric oligonucleotides and vehicle controls were then administered *in vivo* by tail vein injection of the 25 kDa PEI and liver tissue harvested 5 days post-injection. Liver DNA was isolated and subjected to PCR amplification of a 374-nt sequence spanning the targeted nucleotide exchange site, using the same primers as those used with the primary hepatocytes. Following subcloning and transformation of *E. coli*

with the PCR-amplified material, duplicate filter lifts of the transformed colonies were done. The filters were hybridized with the same ^{32}P-labeled 17-mer oligonucleotides specific for either 365-A (wild-type) or 365-C (mutant) and processed post-hybridization. Rats treated with 100 μg of the RIXR chimeric oligonucleotides exhibited an A→C conversion frequency ranging from 13.9% to 18.9%, while those that received a total of 350 μg in two injections showed 40% conversion. In contrast, the vehicle controls showed no hybridization with the 365-C probe. RT-PCR hybridization of isolated RNA indicated A→C conversion frequencies of 26.4% to 28.4% in the high dose livers. The APTT for vehicle-treated rats ranged from 89.7% to 181.9% of control values (131.84% ± 32.89%), while the APTT for the oligonucleotide-treated animals ranged from 48.9% to 61.7% (53.8% ± 4.8%).

The APTT times for a 1/10 dilution of rat test plasma in Hepes buffer (50 mM Hepes/100 mM NaCl/0.02% NaN_3, pH 7.4) were determined for both normal (n = 9) and the double injected animals (n = 3). The factor IX activity of duplicate samples was determined from a log-log standard curve that was constructed from the APTT results for dilution (1:10 to 1:80) of pooled plasma from 12 normal male rats, 6-8 weeks old. The APTT results for the normal rats ranged from 89.7% to 181.9% of the control values (mean = 131.84% ± 32.89%), while the APTT results for the double injected animals ranged from 49.0% to 61.7% (mean 53.8% ± 5.8%). The APTT clotting time in seconds for the normal rats ranged from 60.9 seconds to 81.6 seconds (mean = 71.3 ± 7.3 seconds) while the APTT times ranged from 92.3 seconds to 98.6 seconds (mean = 96.3 ± 2.9 seconds) for the double-infected rats.

Sequence analysis of the mutated factor IX gene in isolated hepatocytes and intact liver

Direct sequencing of the wild-type and mutated genes was performed to confirm the results from the filter hybridizations in both the *in vitro* and *in vivo* studies. At least 10 independent clones hybridizing to either 365-A or 365-C from the intact liver or isolated hepatocytes were analyzed. The results of the sequencing indicated that colonies hybridizing to 365-A (Fig. 6, top panel) exhibited the wild-type IX sequence, i.e. an A at Ser^{365} of the reported cDNA sequence. In contrast, those colonies derived from the factor $RIXR_C$ transfected primary hepatocytes hybridizing to the 365-C oligonucleotide probe

converted to a C at Ser^{365}. The same A→C conversion at Ser^{365} was observed in the clones derived from the transfected rat liver that hybridized with the 17 mer 365-C oligonucleotide probe. The entire 374-nt PCR amplified region of the factor IX gene was sequenced for all the clones and no alteration other than the indicated changes at Ser^{365} was detected. Finally, the start and end points of the 374-nt PCR amplified genomic DNA derived from both the primary hepatocytes and the intact liver corresponded exactly to those of the primers used for the amplification process, indicating that the cloned and sequenced DNA was derived from genomic DNA rather than nondegraded chimeric oligonucleotides.

Table III Percent A→C conversion at Ser^{365} of rat factor IX genomic DNA by colony lift hybridizations

PEI Deliver System		365-C clones	Total clones	A→C (%)
PEI 800 kDa[1]	Concentration			
In vitro	150 nM	24	572	4.2
	300	31	367	8.5
	450	63	502	12.5
Lac-PEI 800 kDa				
In vitro	90	18	337	5.3
	180	34	300	11.3
	270	47	253	18.6
Lac-PEI 25 kDa				
In vitro	90	28	527	5.3
	180	53	417	12.7
	270	60	305	19.7
Lac-PEI 25 kDa[2]	Dose			
In vivo x1	100 μg	24	166	14.5
		71	386	18.4
		50	360	13.9
Lac-PEI 25 kDa				
In vivo x2	350 μg	237	601	39.4
		228	563	40.5
		271	678	40.0

[1]The data shown for the primary hepatocyte transfections represents a mean of two experiments.

[2]The *in vivo* chimeric/PEI complexes were administered in a volume of 300 μl of 5% dextrose by tail vein injection. The results of three animals at each dose are shown individually.

7.3.2 Materials and Methods

In vivo delivery of the chimeric oligonucleotides. Male Sprague-Dawley rats (Harlan Sprague-Dawley, Inc.) (~50 g) were maintained on a standard 12 h light-dark cycle and fed *ad libitum* standard laboratory chow. Vehicle controls and lactosylated 25 kDa PEI at a ratio of 6 equivalents of PEI nitrogen per chimeric phosphate were administered in 300 μl of 5% dextrose (Abdallah, B. et al., "A powerful nonviral vector for *in vivo* gene transfer into the adult mammalian brain: polyethylenimine:, *Human Gene Therapy*, 7, 1947-1954, 1996.). The aliquots were administered by tail vein injection either as a single dose of 100 μg or divided dose of 150 μg and 200 μg on consecutive days. Five days post-injection, liver tissue was removed for DNA and RNA isolation. DNA was isolated as previously described (Kren, B. T., Trembley, J. H. & Steer, C. J., "Alterations in mRNA stability during rat liver regeneration," *Am. J. Physiol.*, 270, G763-G777, 1996) for PCR amplification of exon 8 of the rat factor IX gene. RNA was isolated for RT-PCR amplification of the same region as the genomic DNA using RNAexol and RNAmate (Intermountian Scientific Corp., Kaysville, UT) according to the manufacturer's protocol.

Factor IX activity assay. Blood samples from vehicle (n = 9) and oligonucleotide-treated (n = 3) rats were collected 20 days after the second tail vein injection in 0.1 vol. of 0.105 M sodium citrate/citric acid. After centrifugation at 2,500 x g and then 15,000 x g the resulting plasma was stored at -70°C. The factor IX activity was determined from activated partial thromboplastin time (APTT) assays. Briefly, 50 μl of APTT reagent (DADE, Miami, FL), 50 μl of human factor IX-deficient plasma (George King Biomedical, Overland, KS), and 50 μl of 1/10 dilution of rat test plasma in Hepes buffer (50 mM Hepes/100 mM NaCl/0.02% NaN_3, pH 7.4) were incubated at 37°C for 3 min in an ST4 coagulometer (American Bioproducts, Parsippany, NJ). Clotting was initiated by addition of 50 μl of 33 mM $CaCl_2$ in Hepes buffer. Factor IX activity of duplicate samples was determined from a log-log standard curve constructed from the APTT results for dilution (1:10 to 1:80) of pooled plasma from normal male rats (n = 12).

DNA/RNA isolation and cloning. The cells were harvested by scrapping 48 h after transfection. Genomic DNA larger than 100-150 base pairs was isolated using the high

pure PCR template preparation kit (Boehringer Mannheim, Corp., Indianapolis, IN). RNA was isolated using RNAzol™ B (Tel-Test, Inc., Friendswood, TX), according to the manufacturer's protocol. PCR amplification of a 374-nt fragment of the rat factor IX gene was performed with 300 ng of the isolated DNA from either the primary hepatocytes or liver tissue. The primers were designed as 5'-ATTGCCTTGCTGGAACTGGATAAAC-3' (SEQ ID No. 42) and 5'TTGCCTTTCATTGCACATTCTTCAC-3' (SEQ ID No. 43) (Oligos Etc., Wilsonville, OR) corresponding to nucleotides 433-457 and 782-806, respectively, of the rat factor IX cDNA. Primers were annealed at 59°C for 20 sec, elongation was for 45 sec at 72°C and denaturation proceeded for 45 sec at 94°C. The sample was amplified for 30 cycles using Expand Hi-fidelity™ polymerase (Boehringer Mannheim, Corp.). The PCR amplification products from both the hepatocytes and intact liver factor IX genes were subcloned into the TA cloning vector pCR™2.1 (Invitrogen, San Diego, CA), and the ligated material used to transform frozen competent *E. coli*. To rule out PCR artifacts 300 ng of control DNA was incubated with 0.5, 1.0 and 1.5 μg of the oligonucleotide prior to the PCR-amplification reaction. Additionally, 1.0 μg of the chimeric alone was used as the "template" for the PCR amplification.

RT-PCR amplification was done utilizing the Titian™ one tube RT-PCR system (Boehringer Mannheim, Corp.) According to the manufacturer's protocol using the same primers as those used for the DNA PCR amplification. To rule out DNA contamination, the RNA samples were treated with RQ1 DNase free RNase (Promega Corp., Madison, WI) and RT-PCR negative controls of RNased RNA samples were performed in parallel with the RT-PCR reaction. Each of the PCR reactions were ligated into the same TA cloning vector and transformed into frozen competent *E. coli*.

Colony hybridization and sequencing. Eighteen to 20 h after plating, the colonies were lifted onto MSI MagnaGraph nylon filters, replicated and processed for hybridization according to the manufacturer's recommendation. The filters were hybridized for 24 h with 17 mer oligonucleotide probes 365-A (5'AAGGAGATAGTGGGGGA-3') (SEQ ID No. 44) OR 365-C (5'-AAGGAGATCGTGGGGGA-3') (SEQ ID No. 45) (Life technologies, Inc., Gaithersburg, MD), where the underlined nucleotide is the target for

mutagenesis. The probes were ^{32}P-end-labeled using (γ-^{32}P) ATP (>7,000 Ci/mmol) and T4 polynucleotide kinase (New England Biolabs, Inc., Beverly MA). Hybridizations were performed at 37°C in 2X sodium chloride sodium citrate containing 1% SDS, 5X Denhardt's and 200 μg/ml denatured sonicated fish sperm DNA. After hybridization, the filters were rinsed in 1X sodium chloride sodium phosphate EDTA, 0.5% SDS and then washed at 54°C for 1 h in 50 mM Tris-HCl, pH 8.0 containing 3 M tetramethylammonium chloride, 2 mM EDTA, pH 8.0, 0.1% SDS (Melchior, W. B. & Von Hippel, P. H. "Alteration of the relative stability of dA.dT and dG.dC base pairs in DNA," Proc. Natl. Acad. Sci. USA, **70**, 298-302, 1973.). Autoradiography was performed with NEN®Reflection film at -70°C using an intensifying screen. Plasmid DNA was prepared from colonies identified as hybridizing with 365-A or 365-C using Qiagen miniprep kit (Chatsworth, CA) and subjected to automatic sequencing using the mp13 forward and reverse primers as well as a gene specific primer, 5'GTTGACCGAGCCACATGCCTTAG-3' (SEQ ID No. 46) corresponding to nucleotides 616 to 638 of the rat factor IX cDNA using an ABI 370A sequencer (Perkin-Elmer, Corp., Foster City, CA).

7.4 Examples of CMV Useful for the Reduction of LDL Levels in Humans

A CMV suitable for the modification of Apo B having a sequence comprising the sequence of SEQ ID No: 5 is given below.

Apo B 41/UR (mut→WT) (SEQ ID No. **47**)

```
 u GCGCG gac ccg acc gaa uuc ggu aac ugu au
u                                           u
u                                           u
 u CGCGC CTG GGC TGG CTT AAG CCA TTG ACA Tu
       3' 5'
```

A CMV suitable for the modification of Apo B having a sequence comprising the sequence of SEQ ID No: 12 is given below.

Apo B 5/U88 (mut→WT) (SEQ ID No. **48**)

```
 u GCGCG cug uuc aaa gug uaC GGA TCC ucu uug acu gac gau
u                                                       u
u                                                       u
 u CGCGC GAC AAG TTT CAC ATG CCT AGG AGA AAC TGA CTG CTu
       3' 5'
```

7.5 CORRECTION OF A CRIGLER-NAJJAR-LIKE MUTATION IN THE GUNN RAT

Mutant rats with hyperbilirubinemia, termed Gunn rats, have a single nucleotide deletion in the gene encoding bilirubin-uridinediphosphoglucuronate glucuronosyltransferase (*UGT1A1*). Roy Chowdhury, J., et al., 1991, J. Biol. Chem. **266**, 18294. Human patients with Crigler-Najjar syndrome type I also have mutations of the *UGT1A1* gene, resulting in life-long hyperbilirubinemia and consequent brain damage. Bosma, P.J., et al., 1992, FASEB J. **6**, 2859; Jansen, P.L.M., et al., Progress In Liver Diseases, **XIII**, Boyer, J.L., & Ockner, R.K., editors (W.B. Saunders, Phil. 1995), pp 125-150. The structure of CN3, a CMV designed to correct the Gunn rat mutation is given below.

CN3 (mut→WT) (SEQ ID No. 49)

```
 T GCGCG gg gac uua caG GAC CTT TAC uga ctt cua T
T                                                 T
T                                                 T
 T CGCGC CC CTG AAT GTC CTG GAA ATG ACT GCC GAT T
       3' 5'
```

Gunn rat primary cultured hepatocytes were treated with 150 nM CN3 according to the above protocol except that the carrier was either the negatively charged glycosylated lipid vesicles of section 6.2.2 or a lactosylated-PEI carrier at a ratio of

oligonucleotide phosphate to imine of 1:4 . The results were 8.5% conversion with the negatively charged liposome and 3.6% conversion with lactosylated-PEI carrier.

Gunn rats were injected with 1 mg/Kg of CN3 complexed with either 25 kDa Lac-PEI or complexed with negatively charged Gc lipid vesicles (Gc-NLV) as described above. The rate of gene conversion was determined by cloning and hybridization according to the procedure described for factor IX. The results shown below indicate that between about 15% and 25% of the copies of the *UCT1A1* gene were converted.

Frequency of Insertion of G at nucleotide 1239 of the UGT-1 Gene (In Gunn Rats)

Vehicle	Dosage	G Clones/Total Clones	Frequency (%)
Gc-NLV	1 mg	112/815	15.4
		208/761	27.3
		185/974	18.9
		39/273	14.6[1]
		78/403	19.3[2]
25 kDa PEI (Lactosylated)	1 mg	188/838	22.4
		254/1150	22.1
		245/997	24.6

[1]Initial conversion frequency determined.
[2]Conversion frequency determined 7 days after 70% partial hepatectomy.

A Gunn rat was injected on five successive days with 1mg/Kg of CN3 complexed with 25 kDa Lac-PEI as above. Twenty five days after the final injection the serum bilirubin had declined from 6.2 mg/dl to 3.5 mg/dl and remained at that level for a further 25 days.

7.6 Correction of a Factor IX Mutation in Dog

The Chapel Hill strain of dogs, which has a (G→A)[1477] mutation that results in hemophilia in the animals, was used to obtain primary cultured hepatocytes. Four CMV to correct this mutation have been synthesized.

DIX1 (mut→WT) (SEQ ID No. **50**)

```
 T gcgcg auu caa aga aTT GAC CCT AAT AAT cga ccc cT
T                                                  T
T                                                  T
 T CGCGC TAA GTT TCT TAA CTG GGA TTA TTA GCT GGG GT
       3' 5'
```

DIX2 (mut→WT) (SEQ ID No. **51**)

```
 T gcgcg caa aga auu gAC CCT AAT aau cga cT
T                                        T
T                                        T
 T CGCGC GTT TCT TAA CTG GGA TTA TTA GCT GT
       3' 5'
```

DIX3 (mut→WT) (SEQ ID No. 52)

```
 u gcgcg auu caa aga auu gac ccu aau aau cga ccc cu
u                                                  u
u                                                  u
 u CGCGC TAA GTT TCT TAA CTG GGA TTA TTA GCT GGG Gu
       3' 5'
```

DIX4 (mut→WT) (SEQ ID No. 53)

```
 u gcgcg auu caa aga auu gac ucu aau aau cga ccc cu
u                                                  u
u                                                  u
 u CGCGC TAA GTT TCT TAA CTG GGA TTA TTA GCT GGG Gu
       3' 5'
```

DIX1 differs from DIX3 by the replacement of the intervening DNA segment with 2'-O-methyl RNA and replacement of the tetrathymidine linkers with tetrauracil. DIX 4 differs from DIX3 in that the mutational vector contains a mismatch in the mutator region. In DIX4 the 5' (lower) strand encodes the desired (wild-type) sequence while the 3' (upper) strand has the sequence of the target, i.e., the mutant sequence.

The hepatocytes were treated with 360 nM DIX1 complexed in either 25 kDa Lac-PEI or galactocerebroside-containing aqueous-cored, negatively charged lipid vesicles (Gc-NLV). The results are given in the table below.

Frequency of conversion of A to G at nucleotide 1477 of the Factor IX Gene

(Primary Hepatocytes from the Chapel Hill Strain of Hemophilia B Dogs)

Vehicle	Number of Times Transfected	Concentration	G Clones/Total Clones	Frequency (%)
Gc-NLV	Once	360 nM	30/195	15.44
			30/218	13.76
	Twice		30/118	25.4
Lac-PEI 25 kDa	Once*	360 nM	20/141	14.2
			48/348	13.3
	Twice		21/107	19.6

*RT-PCR on parallel transfected cultures gave an A to G conversion frequency of 11.1%

Each of the DIX2-DIX4 were also tested on primary cultured dog hepatocytes as above. The results showed that DIX2 worked poorly, possibly due to the low (25%) GC percentage. The subsequent experiments the results of DIX3 were about 16% conversion, while a parallel experiments DIX1 gave 10% conversion and the results of DIX4 were about as good as DIX1.

GenBank Sequence References for the Exons of the Human Apolipoprotein B-100 Gene

TABLE II

Exon No.	cDNA Boundary	GenBank Accession No. Sequence Reference
1	126 to 207	M19808
2	208 to 246	M19808
3	247 to 362	M19809
4	363 to 508	M19810
5	509 to 662	M19811
6	663 to 818	M19812
7	819 to 943	M19813
8	944 to 1029	M19813
9	1030 to 1249	M19815
10	1250 to 1477	M19816
11	1478 to 1595	M19818
12	1596 to 1742	M19818
13	1743 to 1954	M19820
14	1955 to 2192	M19820
15	2193 to 2359	M19821
16	2360 to 2561	M19823
17	2562 to 2729	M19824
18	2730 to 2941	M19824
19	2942 to 3124	M19825
20	3125 to 3246	M19825
21	3247 to 3457	M19827
22	3458 to 3633	M19828
23	3634 to 3821	M19828
24	3822 to 3967	M19828
25	3968 to 4341	M19828
26	4342 to 11913	M19828
27	11914 to 12028	M19828
28	12029 to 12212	M19828
29	12213 to 13816	M19828

TABLE I

SEQ ID No.	Sequence (5'→3')	G/C#	NA Change	AA Change	AA	%APOB100	Restriction Site
4	AGTCTGGATGGGIAAGCCGCCCTCA	15	A→T	K→Stop	1701	36.9	None
5	CTGGGCTGGCTTAAGCCATTGACAT	13	C→A	S→TAA	1876	40.8	+CTTAAG
6	GCTCTCTGGGGAIAACATACTGGGC	14	G→T	E→Stop	1921	41.8	None
7	GATGCCGTTGAGIAGCCCCAAGAAT	13	A→T	K→Stop	2047	44.5	None
8	GAGAGGAATCGAIAAACCATTATAG	10	C→T	Q→Stop	2085	45.4	+ATCGAT
9	TGTAAGAAAATAAAAGAGCAGCCCTG	10	C→A	Y→Stop	2110	45.9	None
10	GCAGCCCTGGGAIAACTCCCACAGC	16	A→T	K→Stop	2116	46.0	None
11	GCAAGCTAATGATTAGCTGAATTCATTCAAT	8	T→G	Y→Stop	2124	46.2	+AGCT
12	CAAGTTTCACATGCCIAGGAGAAACTGACTG	11	A→T	K→Stop	2138	46.5	+CCTAGG
13	ATATACAAATTGCATGAGATGATGCCAAAAT	9	T→G	L→Stop	2159	47.0	+CATG
14	AAACTATCTCAACTGIAGACATATATGATAC	8	C→T	Q→Stop	2174	47.3	-CTGCAG
15	GCTAATATTATTGATTAAATCATTGAAATTA	3	G→T	E→Stop	2204	48.0	+TTAA
16	TGATGAGCACTAGCATATCCGTGTA	11	T→G	Y→Stop	2216	48.3	+CTAG
17	CTGCAGCAGCTTIAGAGACACATAC	12	A→T	K→Stop	2270	49.4	-CTTAAG
18	AACAGTGAGCTGIAGTGGCCCGTTC	14	C→T	Q→Stop	2684	58.6	None
19	CAGACTTCCGTTAACCAGAAATCGC	12	T→A	L→Stop	2712	59.2	+GTTAAC
20	AAAGGGTCATGGIAATGGGCCTGCC	14	A→T	K→Stop	2930	64.0	None
21	ACATATATGATAIAATTTGATCAGT	5	C→T	Q→Stop	2180	47.5	Physiologic

Table III

Seq ID No.	Sequence (5'→3')	G/C#	AA Change	NA Change	AA	Gene
22	ATGGAGGACGTGTGCGGCCGCCTGG	18	R⇒C	C→T	112	Apo E
23	GACCTGCAGAAGTGCCTGGCAGTGT	15	R⇒C	C⇒T	158	Apo E
24	GACCTGCAGAAGCGCCTGGCAGTGT	16	C⇒R	T⇒C	158	Apo E
25	TAAGGTCAGGAGTTTGAGACCAGCC	13	NA	A⇒T	-491	Apo E
54	GGCGAGGACATGTGCGACCGGGCGC	19	R⇒C	C⇒T	149	Apo AI
55	GAGATGCGCGACTGCGCGCGCGCCC	20	R⇒C	C⇒T	151	Apo AI
56	CGCGACCGCGCGTGCGCGCATGTGG	20	R⇒C	C⇒T	153	Apo AI
57	AGCGACCAGCTGTGCCAGCGCTTGG	17	R⇒C	C→T	171	Apo AI
58	GAGCTGCGCCAGTGCTTGGCCGCGC	19	R⇒C	C→T	173	Apo AI

WE CLAIM

1. A method of reducing LDL in the blood of a subject comprising altering an Apo B gene of a hepatocyte of the subject such that the transcript of the altered Apo B gene contains an in-frame stop codon whereby the altered gene encodes a protein having at least 1433 amino acids and not more than 3974 amino acids.

2. The method of claim 1, which further comprises the steps of determining the effect on the level of LDL of the alteration of the Apo B genes in the subject and subsequently adjusting the number of altered Apo B genes in the subject.

3. The method of claim 1, wherein the altered gene encodes a protein having at least 1841 amino acids and not more than 2975 amino acids.

4. The method of any one of claims 1-3, wherein the altered gene encodes a protein having a sequence of a fragment of SEQ ID No. 1, which fragment is at least amino acids 1-1841 and not more than amino acids 1-2975.

5. The method of any one of claims 1-4 which comprises administering a recombinagenic oligonucleobase which comprises a first and a second homologous region each having a sequence of at least 10 nucleobases selected from nt 4342-11913 of SEQ ID No: 2, whereby the alteration of the Apo B gene is effected.

6. The method of any one of claims 1-5, wherein the subject's fasting LDL serum cholesterol is reduced to below 140 mg/dl.

7. A composition for the modification of a human Apo B gene comprising an oligonucleobase which oligonucleobase comprises:
   a. a first and a second homologous region that are each at least 8 nucleobases in length and together at least 20 nucleobases in length, which homologous regions are each homologous with a fragment of the sequence of nt 5649-9051 of SEQ ID No. 2, and

b. a heterologous region that is disposed between the first and second homologous region,

such that the introduction of the sequence of the heterologous region into the Apo B gene results in the truncation of the protein encoded thereby.

8. The composition of claim 7, in which the first and the second homologous regions each comprises at least 3 contiguous nucleobase-pairs of hybrid-duplex.

9. The composition of claim 7 or 8, in which the sum of the lengths of the first and second homologous regions is not more than 60 nucleobases in length.

10. The composition of any one of claims 7-9, in which the homologous regions together comprise between 9 and 25 nucleobase pairs of hybrid-duplex.

11. The composition of any one of claims 7-10 , in which the GC fraction of each homologous region is at least 33%.

12. The composition of any one of claims 7-10, in which the GC fraction of a homologous region is at least 50%.

13. The composition of any one of claims 7-10, in which the sequence of the oligonucleobase comprises the sequence of at least a 21 nucleobase fragment of any one of SEQ ID No. 4-21 or the complement thereof.

14. The composition of any one of claims 7-10 , in which the sequence of the oligonucleobase comprises the sequence of at least a 25 nucleobase fragment of any one of SEQ ID No. 4-21 or the complement thereof.

15. The composition of any one of claims 7-14 which further comprises:

a. an aqueous carrier; and

b. a macromolecular carrier selected from the group consisting of

i. an aqueous-cored lipid vesicle, wherein the aqueous core contains the oligonucleobase,

ii. a lipid nanosphere, which comprises a lipophilic salt of the oligonucleobase, and

iii. a polycation having an average molecular weight of between 500 daltons and 1.3 Md wherein the polycation forms a salt with the oligonucleobase.

16. The composition of claim15, in which the macromolecular carrier further comprises a ligand for a clathrin-coated pit receptor.

17. The composition of claim16, in which the clathrin-coated pit receptor is selected from the group consisting of the receptors for transferrin, nicotinic acid, carnitine, insulin and insulin like growth factor-1.

18. The composition of claim 16, in which the clathrin-coated pit receptor is an asialoglycoprotein receptor.

19. The composition of any one of claims 15-18, in which the macromolecular carrier is a negatively charged, aqueous-cored lipid vesicle.

20. The composition of any one of claims16-18, in which the aqueous-cored lipid vesicle further comprises a cerebroside.

21. The composition of any one of claims 15-20, in which the aqueous-cored lipid vesicle comprises dioleoylphosphatidylcholine and dioleoylphosphatidylserine.

22. The composition of any one of claims 16, 18, 19 and 21 wherein the macromolecular carrier is an aqueous-cored lipid vesicle that comprises a fusigenic F-protein.

23. The composition of any one of claims 15-22 , in which the oligonucleobase comprises:

a. a first and a second homologous region that are together at least 16 and not more than 60 nucleobases in length, which regions are homologous with a target gene of a mammal; and

b. a heterologous region that is disposed between the first and second homologous region and is at least 1 and not more than 20 nucleobases in length, which is heterologous with the target gene and which contains the alteration.

24. A method of treatment and/or prophylaxis in a subject comprising altering an Apo E gene of a hepatocyte of the subject by introducing a substitution selected from the group $(Arg \rightarrow Cys)^{112}$, $(Arg \rightarrow Cys)^{158}$ and $(Cys \rightarrow Arg)^{158}$.

25. The method of claim 24, wherein the subject is homozygous for Apo E4 and the alteration comprises the substitution $(Arg \rightarrow Cys)^{112}$.

26. The method of claim 24 or 25, which comprises administering a chimeric mutational vector having a sequence which comprises SEQ ID No: 22.

27. The method of claim 24, wherein the treatment or prophylaxis comprises reducing the subject's fasting serum LDL cholesterol level and the alteration comprises the substitution $(Arg \rightarrow Cys)^{158}$.

28. The method of claim 24 or 27, which comprises administering a chimeric mutational vector having a sequence which comprises SEQ ID No: 23.

29. The method of claim 24, wherein the subject suffers from Type III hyperlipidemia and the alteration comprises the substitution $(Cys \rightarrow Arg)^{158}$.

30. The method of claim 24 or 29, which comprises administering a recombinagenic oligonucleobase having a sequence which comprises SEQ ID No: 24.

31. A composition for the alteration of a human Apo E gene comprising a recombinagenic oligonucleobase having a sequence comprising the sequence of at least a 21 nucleobase fragment of any one of SEQ ID No. 22 - 25 or the complement thereof.

32. A method of ameliorating atherosclerosis in a subject comprising altering an Apo A1 gene of a hepatocyte of the subject such that the altered Apo A1 protein forms dimers.

33. The method of claim 32, which further comprises the steps of determining the effect on the level of HDL of the alteration of the Apo A1 genes in the subject and subsequently adjusting the number of altered Apo A1 genes in the subject.

34. The method of claim 32, wherein the altered gene encodes a protein having a cysteine for arginine substitution at a position selected from the group consisting of residue 149, 151, 153, 171 and 173.

35. The method of claim 34, wherein the method comprises the administration of a recombinagenic oligonucleobase having a sequence comprising the sequence of at least 20 nucleotides of SEQ ID No. 54, No. 55, No. 56, No. 57 and No. 58.

36. The method of claim 35, which comprises the step of administering to the subject a composition comprising:
    a) the recombinagenic oligonucleobase;
    b) an aqueous carrier; and
    c) a macromolecular carrier selected from the group consisting of

(i) an aqueous-cored lipid vesicle, wherein the aqueous core contains the oligonucleobase,

(ii) a lipid nanosphere, which comprises a lipophilic salt of the oligonucleobase, and

(iii) a polycation having an average molecular weight of between 500 daltons and 1.3 Md wherein the polycation forms a salt with the oligonucleobase.

37. The method of claim 36, wherein the macromolecular carrier is a negatively charged, aqueous-cored lipid vesicle.

38. The method of claim 36, wherein the macromolecular carrier further comprises a ligand for a clathrin-coated pit receptor.

39. The method of claim 38, wherein the clathrin-coated pit receptor is an asialoglycoprotein receptor.

40. The method of claim 32, which comprises administering to the subject a composition comprising:

a) a recombinagenic oligonucleobase;

b) an aqueous carrier; and

c) a macromolecular carrier selected from the group consisting of

(i) an aqueous-cored lipid vesicle, wherein the aqueous core contains the oligonucleobase,

(ii) a lipid nanosphere, which comprises a lipophilic salt of the oligonucleobase, and

(iii) a polycation having an average molecular weight of between 500 daltons and 1.3 Md wherein the polycation forms a salt with the oligonucleobase.

41. The method of claim 40, wherein the macromolecular carrier is a negatively charged, aqueous-cored lipid vesicle.

42. The method of claim 40, wherein the macromolecular carrier further comprises a ligand for a clathrin-coated pit receptor.

43. The method of claim 42, wherein the clathrin-coated pit receptor is an asialoglycoprotein receptor.

44. A composition for the modification of a human Apo A1 gene comprising an oligonucleobase which oligonucleobase contains a sequence which comprises a fragment having the sequence selected from the group consisting of SEQ ID No. 54, No. 55, No. 56, No.57 and No. 58.

45. The composition of claim 44, which further comprises:
    a) an aqueous carrier; and
    b) a macromolecular carrier selected from the group consisting of
        (i) an aqueous-cored lipid vesicle, wherein the aqueous core contains the oligonucleobase,
        (ii) a lipid nanosphere, which comprises a lipophilic salt of the oligonucleobase, and
        (iii) a polycation having an average molecular weight of between 500 daltons and 1.3 Md wherein the polycation forms a salt with the oligonucleobase.

46. The composition of claim 45, in which the macromolecular carrier further comprises a ligand for a clathrin-coated pit receptor.

47. The composition of claim 46, in which the clathrin-coated pit receptor is selected from the group consisting of the receptors for transferrin, nicotinic acid, carnitine, insulin and insulin like growth factor-1.

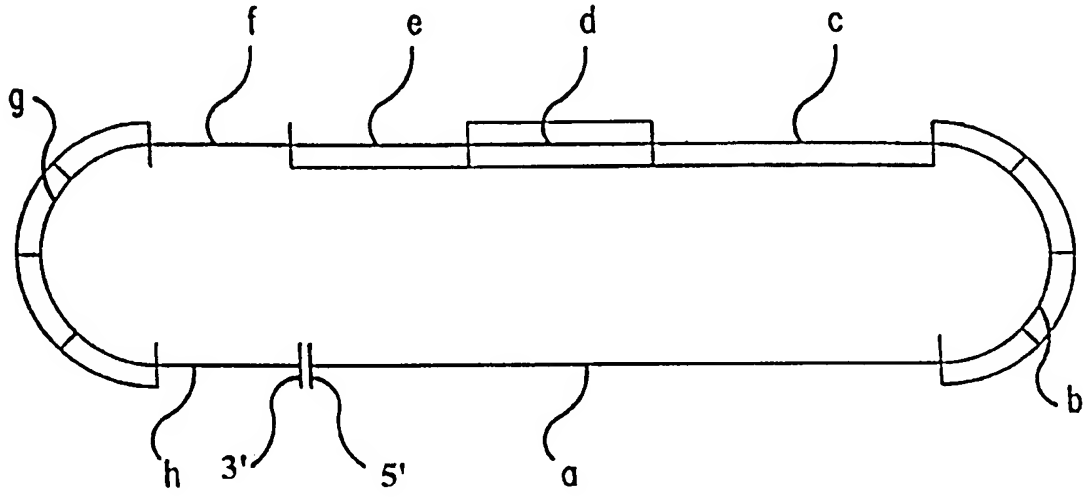
48. The composition of claim 46, in which the clathrin-coated pit receptor is an asialoglycoprotein receptor.

49. The composition of any one of claims 45-48, in which the macromolecular carrier is a negatively charged, aqueous-cored lipid vesicle.

50. The composition of any one of claims 46-48, in which the aqueous-cored lipid vesicle further comprises a cerebroside.

51. The composition of any one of claims 45-50, in which the aqueous-cored lipid vesicle comprises dioleoylphosphatidylcholine and dioleoylphosphatidylserine.

52. The composition of any one of claims 46, 48, 49 and 51 wherein the macromolecular carrier is an aqueous-cored lipid vesicle that comprises a fusigenic F-protein.



RIBO- OR DEOXYRIBO-TYPE

DEOXYRIBO-TYPE

RIBO-TYPE

LINKER

SEGMENT BOUNDARIES

FIG.1

FIG. 2A

```
1     cctatccctgggggaggggcgggacaggggagccctataattggacaagtctgggatccttgagtcctACTCAGCCCCAG

83    CGGAGGTGAAGGACGTCCTTCCCCAGGAGCCGgtgagaagcgcagtcgggggcacggggatgagctcaggggcctctagaaa

165   gagctgggaccctgggaagccctggcctccaggtagtctcaggagagctactcggggtcgggcttggggagaggaggagcgg

247   gggtgaggcaagcagcaggggactggacctgggaagggctgggcagcagagacgacccgacccgctagaaggtggggtgggg

329   agagcagctggactgggatgtaagccatagcaggactccacgagttgtcactatcattatcgagcacctactgggtgtcccc

411   agtgtcctcagatctccataactggggagccaggggcagcgacacggtagctagccgtcgattggagaactttaaaatgagg

493   actgaattagctcataaatggaacacggcgcttaactgtgaggttggagcttagaatgtgaagggagaatgaggaatgcgag

575   actgggactgagatggaaccggcggtggggaggggtgggggatggaatttgaaccccgggagaggaagatggaatttttct

657   atggaggccgacctggggatggggagataagagaagaccaggagggagttaaatagggaatgggttgggggcggcttggtaa

739   atgtgctgggattaggctgttgcagataatgcaacaaggcttggaaggctaacctggggtgaggccgggttgggggcgctgg

821   gggtgggaggagtcctcactggcggttgattgacagtttctccttccccagACTGGCCAATCACAGGCAGGAAGATGAAGGT
                                                                          M  K  V

903   TCTGTGGGCTGCGTTGCTGGTCACATTCCTGGCAGgtatgggggcggggcttgctcggttcccccccgctcctcccccctcca
        L  W  A  A  L  L  V  T  F  L  A

985   tcctcacctcaacctcctggccccattcagacagaccctgggccccctcttctgaggcttctgtgctgcttcctggctctga

1067  acagcgatttgacgctctctgggcctcggtttcccccatccttgagataggagttagaagttgttttgttgttgtttgt

1149  tgttgttgttttgttttttttgagatgaagtctcgctctgtcgcccaggctggagtgcagtggcgggatctcggctcactgca

1231  agctccgcctcccaggtccacgccattctcctgcctcagcctcccaagtagctgggactacaggcacatgccaccacacccg

1313  actaacttttttgtattttcagtagagacggggtttcaccatgttggccaggctggtctggaactcctgacctcaggtgatc
```

FIG. 2B

```
1395  tgcccgtttcgatctcccaaagtgctgggattacaggcgtgagccaccgcacctggctgggagttagaggtttctaatgcat

1477  tgcaggcagatagtgaataccagacacggggcagctgtgatctttattctccatcacccccacacagccctgcctggggcac

1559  acaaggacactcaatacatgcttttccgctgggccggtggctcacccctgtaatcccagcactttgggaggccaaggtggga

1641  ggatcacttgagcccaggagttcaacaccagcctgggcaacatagtgagaccctgtctctactaaaaatacaaaaattagcc

1723  aggcatggtgccacacacctgtgctctcagctactcaggaggctgaggcaggaggatcgcttgagcccagaaggtcaaggtt

1805  gcagtgaaccatgttcaggccgctgcactccagcctgggtgacagagcaagaccctgtttataaatacataatgctttccaa

1887  gtgattaaaccgactcccccctcaccctgcccaccatggctccaaagaagcatttgtggagcaccttctgtgtgccccctagg

1969  tagctagatgcctggacggggtcagaaggaccctgacccgaccttgaacttgttccacacagGATGCCAGGCCAAGGTGGAG
                                                                    G  C  Q  A  K  V  E

2051  CAAGCGGTGGAGACAGAGCCGGAGCCCGAGCTGCGCCAGCAGACCGAGTGGCAGAGCGGCCAGCGCTGGGAACTGGCACTGG
       Q  A  V  E  T  E  P  E  P  E  L  R  Q  Q  T  E  W  Q  S  G  Q  R  W  E  L  A  L

2133  GTCGCTTTTGGGATTACCTGCGCTGGGTGCAGACACTGTCTGAGCAGGTGCAGGAGGAGCTGCTCAGCTCCCAGGTCACCCA
       G  R  F  W  D  Y  L  R  W  V  Q  T  L  S  E  Q  V  Q  E  E  L  L  S  S  Q  V  T  Q

2215  GGAACTGAGgtgagtgtccccatcctggcccttgaccctcctggtgggcggctatacctccccaggtccaggtttcattctg
         E  L  R

2297  cccctgtcgctaagtcttgggggcctgggtctctgctggttctagcttcctcttcccatttctgactcctggctttagctc

2379  tctggaattctctctctcagctttgtctctctctcttccctctgactcagtctctcacactcgtcctggctctgtctctgt

2461  ccttccctagctcttttatatagagacagagagatggggtctcactgtgttgcccaggctggtcttgaacttctgggctcaa

2543  gcgatcctcccgcctcggcctcccaaagtgctgggattagaggcatgagcaccttgcccggcctcctagctccttcttcgtc

2625  tctgcctctgccctctgcatctgctctctgcatctgtctctgtctccttctctcggcctctgccccgttccttctctccctc

2707  ttgggtctctctggctcatccccatctcgcccgccccatcccagcccttctcccccgcctccccactgtgcgacaccctccc
```

FIG. 2C

```
2789  gccctctcggccgcagGGCGCTGATGGACGAGACCATGAAGGAGTTGAAGGCCTACAAATCGGAACTGGAGGAACAACTGAC
                        A  L  M  D  E  T  M  K  E  L  K  A  Y  K  S  E  L  E  E  Q  L  T

2871  CCCGGTGGCGGAGGAGACGCGGGCACGGCTGTCCAAGGAGCTGCAGGCGGCGCAGGCCCGGCTGGGCGCGGACATGGAGGAC
        P  V  A  E  E  T  R  A  R  L  S  K  E  L  Q  A  A  Q  A  R  L  G  A  D  M  E  D

2953  GTGTGCGGCCGCCTGGTGCAGTACCGCGGCGAGGTGCAGGCCATGCTCGGCCAGAGCACCGAGGAGCTGCGGGTGCGCCTCG
        V  C  G  R  L  V  Q  Y  R  G  E  V  Q  A  M  L  G  Q  S  T  E  E  L  R  V  R  L

3035  CCTCCCACCTGCGCAAGCTGCGTAAGCGGCTCCTCCGCGATGCCGATGACCTGCAGAAGCGCCTGGCAGTGTACCAGGCCGG
        A  S  H  L  R  K  L  R  K  R  L  L  R  D  A  D  D  L  Q  K  R  L  A  V  Y  Q  A  G

3117  GGCCCGCGAGGGCGCCGAGCGCGGCCTCAGCGCCATCCGCGAGCGCCTGGGGCCCCTGGTGGAACAGGGCCGCGTGCGGGCC
          A  R  E  G  A  E  R  G  L  S  A  I  R  E  R  L  G  P  L  V  E  Q  G  R  V  R  A

3199  GCCACTGTGGGCTCCCTGGCCGGCCAGCCGCTACAGGAGCGGGCCCAGGCCTGGGGCGAGCGGCTGCGCGCGCGGATGGAGG
        A  T  V  G  S  L  A  G  Q  P  L  Q  E  R  A  Q  A  W  G  E  R  L  R  A  R  M  E

3281  AGATGGGCAGCCGGACCCGCGACCGCCTGGACGAGGTGAAGGAGCAGGTGGCGGAGGTGCGCGCCAAGCTGGAGGAGCAGGC
        E  M  G  S  R  T  R  D  R  L  D  E  V  K  E  Q  V  A  E  V  R  A  K  L  E  E  Q  A

3363  CCAGCAGATACGCCTGCAGGCCGAGGCCTTCCAGGCCCGCCTCAAGAGCTGGTTCGAGCCCCTGGTGGAAGACATGCAGCGC
          Q  Q  I  R  L  Q  A  E  A  F  Q  A  R  L  K  S  W  F  E  P  L  V  E  D  M  Q  R

3445  CAGTGGGCCGGGCTGGTGGAGAAGGTGCAGGCTGCCGTGGGCACCAGCGCCGCCCCTGTGCCCAGCGACAATCACTGAACGC
        Q  W  A  G  L  V  E  K  V  Q  A  A  V  G  S  S  A  A  P  V  P  S  D  N  H

3527  CGAAGCCTGCAGCCATGCGACCCCACGCCACCCCGTGCCTCCTGCCTCCGCGCAGCCTGCAGCGGGAGACCCTGTCCCCGCC

3609  CCAGCCGTCCTCCTGGGGGTGGACCCTAGTTTAATAAAGATTCACCAAGTTTCACGCatctgctggcctcccctgtgatttc

3691  ctctaagccccagcctcagtttctctttctgcccacatactgccacacaatttctcagccccctcctctccatctgtgtctgt

3373  gtgtatctttctctctgccctttttttttttttt
```

FIG. 3A

```
   1 ctaaagaaga gcactggtgg gaggacaggg cgggggaagg gggaggggag tgaagtagtc
  61 tccctggaat gctggtggtg ggggaggcag tctccttggt ggaggagtcc cagcgtccct
 121 cccctcccct cctctgccaa cacaatggac aatggcaact gcccacacac tcccatggag
 181 gggaagggga tgagtgcagg gaaccccgac cccacccggg agacctgcaa gcctgcagca
 241 ctcccctccc gcccccactg aacccttgac ccctgccctg cacgccccgc agcttgctgt
 301 ttgcccactc ctatttgccc agtcccaggg acagagctga tccttgaact cttaagttcc
 361 acattgccag gaccagtgag cagcaacagg gccagggctg ggcttatcag cctcccagcc
 421 cagaccctgg ctgcagacat aaataggccc tgcaagagct ggctgcttag agactgcgag
 481 aaggaggtgc gtcctgctgc ctgccccggc actctggctc cccagctcaa ggttcaggcc
 541 ttgccccagg ccgggcctct gggtacctga ggtcttctcc cgctctgtgc ccttctcctc
 601 acctggctgc aatgagtggg ggagcacggg gcttctgcat gctgaaggca ccccactcag
 661 ccaggccctt cttctcctcc aggtccccca cggcccttca ggatgaaagc tgcggtgctg
 721 accttggccg tgctcttcct gacgggtagg tgtcccctaa cctaggagcc aaccatcggg
 781 gggctttctc cctaaatccc cgtggcccac cctcctgggc agaggcagca ggtttctcac
 841 tggcccccctc tcccccacct ccaagcttgg cctttcggct cagatctcag cccacagctg
 901 gcctgatctg ggtctcccct cccaccctca gggagccagg ctcggcattt ctggcagcaa
 961 gatgaacccc cccagagccc ctgggatcga gtgaaggacc tggccactgt gtacgtggat
1021 gtgctcaaag acagcggcag agactatgtg tcccagtttg aaggctccgc cttgggaaaa
1081 cagctaaagt aaggacccag cctggggttg agggcagggg caggggggcag aggcctgtgg
1141 gatgatgttg aagccagact ggccgagtcc tcacctaata tctgatgagc tgggccccac
1201 agatggtctg gatggagaaa ccggaatgga tctccaggca gggtcacagc ccatgtcccc
1261 tgcaaaggac agaccagggc tgcccgatgc gtgatcacag agccacattg tgcctgcaag
1321 tgtagcaagc ccctttccct tcttcaccac ctcctctgct cctgcccagc aagactgtgg
1381 gctgtcttcg gagaggagaa tgcgctggag gcatagaagc gaggtccttc aagggcccac
```

FIG. 3B

```
1441 tttggagacc aacgtaactg ggcaccagtc ccagctctgt ctccttttta gctcctctct
1501 gtgcctcggt ccagctgcac aacggggcat ggcctggcgg ggcaggggtg ttggttgaga
1561 gtgtactgga aatgctaggc cactgcacct ccgcggacag gtgtcaccca gggctcaccc
1621 ctgataggct ggggcgctgg gaggccagcc ctcaaccctt ctgtctcacc ctccagccta
1681 aagctccttg acaactggga cagcgtgacc tccaccttca gcaagctgcg cgaacagctc
1741 ggccctgtga cccaggagtt ctgggataac ctggaaaagg agacagaggg cctgaggcaa
1801 gagatgagca aggatctgga ggaggtgaag gccaaggtgc agccctacct ggacgacttc
1861 cagaagaagt ggcaggagga gatggagctc taccgccaga aggtggagcc gctgcgcgca
1921 gagctccaag agggcgcgcg ccagaagctg cacgagctgc aagagaagct gagcccactg
1981 ggcgaggaga tgcgcgaccg cgcgcgcgcc catgtggacg cgctgcgcac gcatctggcc
2041 ccctacagcg acgagctgcg ccagcgcttg gccgcgcgcc ttgaggctct caaggagaac
2101 ggcggcgcca gactggccga gtaccacgcc aaggccaccg agcatctgag cacgctcagc
2161 gagaaggcca agcccgcgct cgaggacctc cgccaaggcc tgctgcccgt gctggagagc
2221 ttcaaggtca gcttcctgag cgctctcgag gagtacacta agaagctcaa cacccagtga
2281 ggcgcccgcc gccgcccccc ttcccggtgc tcagaataaa cgtttccaaa gtgggaagca
2341 gcttctttct tttgggagaa tagaggggg tgcggggaca tccgggggag cccgggaggg
2401 gcctttggcc ctggagcagg gacttcctgc cggatctcaa caactccgtg cccagactgg
2461 acgtcttagg gccaagatcg acgttggagg acctgctgga cgcntggctg cttacgagtg
2521 agggagtaga gtctgcctta gcaaggctca agtagaaagg aagtcacagc ggacnaggca
2581 aagccacaga caatccaagg ccaggtgccc tgaaaggggc tcaaacaagg cctgcagcct
2641 gtctgaggcg ggccaggaaa cagcggttgc tttagctggg agcagtcggg ttccccgtcc
2701 ccagaggtgt gtccgtatag agccttctcc agcccagccg ctgtcagcgg gcgggacgga
2761 gcggggcgcc tcagggagcc agccactggg attggggttt ggtcccgggt gcaagtgaag
2821 cgcttggagt ttgcgcctgt cctcctttac taattcaaaa acctctcaaa cagacacttc
```

FIG. 3C

```
2881 ccttttcttc tcacaaggcc agtatccccc tcccactact cccatcccgc ccagaaacag
2941 ccgcggcttc ctcaggcaca gcagtggaag ccagtcctcc acccccctgcg gctccatgcc
3001 atgccacccc ctctttctgc cagccctggc agaagctggc ctgagtaaga aaattcacca
3061 ccacctcttg caggtacatt tttatttcca agatgctctc atatctgtgc tctcactgca
3121 tcctcccttc cccacatcct ggctagattg ccatcagacg cagagcatgg atgaggacac
3181 tgaagcctgg acctgtgacg tcgcttgccc agtgaacagc aggatgggct aggcagcgct
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3301 ggtggctcaa gcctgtaatc ccagcacttt gggaggctga ggtgggcgta tcacttcagg
3361 gccaggagtt tgagaccagc ctggccaata tggtgaaacc ctgtctctac taaaaataca
3421 aaaaaaaaat cagctggcat ggtggcttgc acccgtaatc ccagctacta ggaaggctga
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3601 agtgtgtggc ctggcactca agttcacatg ggtgtgcagg catgcctgtg tattctcaca
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3721 atggcttcac agatctggca gtgccttccc taccctctct ctgcagggcc ttttgccccc
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3841 ctaccctagg taaaggagtg ggctttttag ttcctaacct tccagagcta caacagcagt
3901 catccagcca ggtctgggtg ggaacatttt ctagatacgg gtgctgagat ctctcagccc
3961 agagagaagc cctggggaat tttcagagag aaagcagtct ccaggtgggg ctggatgtac
4021 tgatgccact gagatctgta aaggagtccc taacacctga cataggagtg acaaaactgt
4081 tttctgcacc aactgagcag aatacacgca gctgacctgg gctcaaggtc tggccctgcc
4141 acgtgctggc tctgtgatgc tggccaagtg ccttcgcctc tccgggccac agtttttga
4201 tctgaagagt ggagccctac tcaagccatc tgcagctctc gggctctctg acctgacatc
4261 tttcgggtgg tggggacaca aaggaagcag cctctattgg gagaccttgt gctttttttt
```

FIG. 3D

```
4321 ggtcccagga cactgccccc caccactcca gtccggtccc aagggcccag tcagctcaac
4381 tgtaatcatg acaacattga tcaagcatct ttacgtgcag gtgctgtgcc aaacggttcg
4441 aacgctctct catttcaatc tcacggcaaa cctacggtgg agggggtacg gttgtatcca
4501 ctttacatgt aagaaactga ggctgatatc aagtggtgga gccaagaata gtgcctcgtt
4561 gcatcttact ccaacctcta gcccatccgg cctcctccct tcacgtgcgc ctaagagggc
4621 taggtggcct ggatagggga ggtcagctcc acagttttga gtaaacacac acagtctcaa
4681 ctctgatgac acttaagtgc caggcatagt ggctggcatg gggcacacac tcaagtcatg
4741 ttgtgcagca cctaacagtt tatcaaagta tcagcaaact tattgtcctg tttgaccttc
4801 cgcacaaagc tgtcaaggaa ggcagggtac ggagggtgat tcctaccttа gagatgaaga
4861 aactgaggcc cagagactag cccagctacc agaaggtgga tagagcgctg gcctccatgc
4921 ctgcctgacc tggagtctgt ccagtgccca cccacagaac agcctcggcc ctttcccatg
4981 cccagacaca gatggcacac ttgcgacggc ccactcatag cagcttcttg tccagcttta
5041 ttgggaggcc agcatgcctg gaggggggcc aggcatgagg tggggtagga gagcactgag
5101 aatactgtcc cttttaagca acctacaggg gaagccctgg agattgcagg acccaaggag
5161 ctggcaggat ggataggcag gtggacttgg ggtattgagg tctcaggcag ccacggctga
5221 agttggtctg acctcagggt ccaaatccca gaactcagag aacttgtcct taacggtgct
5281 ccagtagtct ttcagggaac tgaagccatc ggtcacccag cccctaaatc agtcagggga
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5581 ggcctgaggc acaaagtgac agggtgggga gatgtgaaag gtcaaggctg tcattgtttt
5641 ctgtgcaaac agcaccacct ggagttgcac aacctggtgg ctctgagcag ggtaggacag
5701 agggaggcag cctctcattt ggaaagtcat tggaggattg attagttgtg tgatctgggg
```

FIG. 3E

```
5761 caggtcacct aatcgctctg agcctcaatt ttctcatctg caaagtgaga aaataacacc
5821 taccccaaag ccggttctgg ggactaagaa tgtttatgaa cacctctgct atgccagcta
5881 atgccagtca gggtgaggtg gagaagggag taggggagag gagagtactg atgggcaggg
5941 gcaggatggg agaggaagga cacacttctg ctcacactgg ccccaggtct tagaacaaag
6001 cagaagcact cacgggcttg aattgggtca ggtggggcct cccaaggcaa acccctagcc
6061 cagggtcccc aacccccaag tcatagatag gtactggtcc atggactgtt aggagccggg
6121 ctgcacagca cgaagtgagt ggtgggtgag tgagcaatac gcctgagctc cgcctcctgt
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6241 agggatctag gtggagggct ccttatgaga atctaatgcc tgatgatctg aggtggaaca
6301 gcttcatccc aaaaccatcc ccgctggccc atggaaaaat tgtccaccac aaaactggtc
6361 cctggtgcca aaaaggctga ggaccactgc tctagccaga ccttcagaaa aggaaaatgg
6421 ggccaggcgc agtggctcgt gcctgtaatc ccagcacttt gggaggccga ggcaggagga
6481 tcccctgagg tcaggaattc aagaccaacc tggccaacat ggtgaaaccc catctctgct
6541 aaaaatacaa aaattagctg ggtgtggtgg cgcgtgcctg taatcccagc tacttgggag
6601 gctgaggcag gagaatgggt tgaacccggg agacggaggt tgcagtgagc cgagatggca
6661 ccactgcact ccagcctggg tgacagaggg agactccatt aaaaaaaaaa gaagaagaaa
6721 gaaagagaga aaggaagaga gggaaagaaa gaaaaaggaa agaaagaaag aaagagaaag
6781 aaagaaaaga aagaaagaaa ggaaagaaag aaaggaaaga aagaaagaaa gaaagaaaga
6841 aagaaagaaa gaaagaaaga aagaaaaata gaaagagaaa gaaagggaaa ggaaaggaaa
6901 atggaaatga gggctaaaac ggcgcggccc taggactgct ccgggagaaa ggagggacct
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7021 aggaaagagg aggctgaaga gcacaggccc aggggaggct ggagcacctc cattccattg
7081 ttgggatctc accagggcag gggcgggtgg gaatggaggc agctggcagg ggggatgggg
7141 agggaggcca gcgggtgtac ctggcctgct gggccacctg ggactcctgc acgctgctca
```

FIG. 3F

7201 gtgcatcctt ggcggtcttg gtggcgtgct tcatgtagcc ctgcatgaag ctgagaaggg
7261 aggcatcctc ggcctctgaa gctcctgagg aaagagcagg gctgagtggg gtggatcggc
7321 ctctggacga gccctggggc tcctgcttga ccacccattg gactgggatc cccaagttgc
7381 ctccaccctg cccccagccc agtcccacca agtgcttacg ggcagaggcc aggagcgcca
7441 ggagggcaac aacaaggagt acccggggct gcatggcacc tctgttcctg caaggaagtg
7501 tcctgtgagg ggcaccccag gtccccatgc ctctggaccc ctccctgggg aggtggcgtg
7561 gcccctaagg tagaacctta gctgggtctg ccagaaggag taggggccgg ctcctgctca
7621 tacgggctct cagaaggggg actggtgagc ggcgagggat cgaggcccaa aggaggtggg
7681 tgggatggag cagaaaaccc accagactga acatcaaggc actgcggtct ggactgatct
7741 ccgtccagtc cagccaacat gctgtgtgtc tttgggtgat ttctggccct cttccaggcc
7801 tcagtttccc tgtctggggt aggactgggc tgtctaaggc ttcctccagc cctaagcctg
7861 aagaatgagg ggggaacctg cacttggagc cacttccagc cccacccccc atgtagcttt
7921 gggcaagtga cacctcccgg gcctccatgt tcttcaggtt atgatgaggg gtgggggca
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8341 tgcagcccag atgagctcag gaactggggg tgggcctggg gagcctcatt ccttcctagc
8401 tgactggctc cccagggaga ggctggtgag aggggaaatg gcttggacat aggccagcgc
8461 tcgggcccat ctcagccttt cacactggaa tttcaggccc ctccctccac cagccccaag
8521 ccctgaacac agcctggagt agaggggtga ggggcttctt cagacttgag aacaagtggg
8581 tggcttgggc tgggggtgt ttggagtaaa ggcacagaag accgggcatc agtggctcgc

FIG. 3G

```
8641 aaggcctggt tacagggctg agctcacagc ccctcccagc acctccatct ctgggtttca
8701 atccaggcag gcgagtgctg ctcacccagc agccccccac ccgcccccac cctgtgtgcc
8761 ccaccgccgc ctccccctga gtgtagggca ggggttggtg gagaagcgca aggccgctca
8821 gagcccgagg cctttgcccc tccctccacc aggctcccta ttttgccctc tggacccact
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8941 ttcctcagtg gggctggggg aagagc
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SEQUENCE LISTING

<110> 1. Steer, Clifford J.
2. Kren, Betsy T.
3. Bandyopadhyay, Paramita
4. Roy-Chowdhury, Jayanta

<120> Methods and Compounds for the Genetic Treament of Hyperlipidemia

<130> 7991-033-228

<150> 60/074,497
<151> 1998-02-12

<150> 09/108,006
<151> 1998-06-30

<160> 59

<170> FastSEQ for Windows Version 3.0

<210> 1
<211> 4563
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<400> 1

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Leu Leu Leu Leu Leu Leu Ala Gly Ala Arg Ala Glu Glu Glu Met Leu
            20                  25                  30
Glu Asn Val Ser Leu Val Cys Pro Lys Asp Ala Thr Arg Phe Lys His
        35                  40                  45
Leu Arg Lys Tyr Thr Tyr Asn Tyr Glu Ala Glu Ser Ser Ser Gly Val
    50                  55                  60
Pro Gly Thr Ala Asp Ser Arg Ser Ala Thr Arg Ile Asn Cys Lys Val
65                  70                  75                  80
Glu Leu Glu Val Pro Gln Leu Cys Ser Phe Ile Leu Lys Thr Ser Gln
                85                  90                  95
Cys Ile Leu Lys Glu Val Tyr Gly Phe Asn Pro Glu Gly Lys Ala Leu
            100                 105                 110
Leu Lys Lys Thr Lys Asn Ser Glu Glu Phe Ala Ala Ala Met Ser Arg
        115                 120                 125
Tyr Glu Leu Lys Leu Ala Ile Pro Glu Gly Lys Gln Val Phe Leu Tyr
    130                 135                 140
Pro Glu Lys Asp Glu Pro Thr Tyr Ile Leu Asn Ile Lys Arg Gly Ile
145                 150                 155                 160
Ile Ser Ala Leu Leu Val Pro Pro Glu Thr Glu Glu Ala Lys Gln Val
                165                 170                 175
Leu Phe Leu Asp Thr Val Tyr Gly Asn Cys Ser Thr His Phe Thr Val
            180                 185                 190
Lys Thr Arg Lys Gly Asn Val Ala Thr Glu Ile Ser Thr Glu Arg Asp
```

```
Lys Ser Val Ser Ile Pro Ser Leu Asp Pro Ala Ser Ala Lys Ile Glu
                645                 650                 655
Gly Asn Leu Ile Phe Asp Pro Asn Asn Tyr Leu Pro Lys Glu Ser Met
            660                 665                 670
Leu Lys Thr Thr Leu Thr Ala Phe Gly Phe Ala Ser Ala Asp Leu Ile
        675                 680                 685
Glu Ile Gly Leu Glu Gly Lys Gly Phe Glu Pro Thr Leu Glu Ala Leu
    690                 695                 700
Phe Gly Lys Gln Gly Phe Phe Pro Asp Ser Val Asn Lys Ala Leu Tyr
705                 710                 715                 720
Trp Val Asn Gly Gln Val Pro Asp Gly Val Ser Lys Val Leu Val Asp
                725                 730                 735
His Phe Gly Tyr Thr Lys Asp Asp Lys His Glu Gln Asp Met Val Asn
            740                 745                 750
Gly Ile Met Leu Ser Val Glu Lys Leu Ile Lys Asp Leu Lys Ser Lys
        755                 760                 765
Glu Val Pro Glu Ala Arg Ala Tyr Leu Arg Ile Leu Gly Glu Glu Leu
    770                 775                 780
Gly Phe Ala Ser Leu His Asp Leu Gln Leu Leu Gly Lys Leu Leu Leu
785                 790                 795                 800
Met Gly Ala Arg Thr Leu Gln Gly Ile Pro Gln Met Ile Gly Glu Val
                805                 810                 815
Ile Arg Lys Gly Ser Lys Asn Asp Phe Phe Leu His Tyr Ile Phe Met
            820                 825                 830
Glu Asn Ala Phe Glu Leu Pro Thr Gly Ala Gly Leu Gln Leu Gln Ile
        835                 840                 845
Ser Ser Ser Gly Val Ile Ala Pro Gly Ala Lys Ala Gly Val Lys Leu
    850                 855                 860
Glu Val Ala Asn Met Gln Ala Glu Leu Val Ala Lys Pro Ser Val Ser
865                 870                 875                 880
Val Glu Phe Val Thr Asn Met Gly Ile Ile Ile Pro Asp Phe Ala Arg
                885                 890                 895
Ser Gly Val Gln Met Asn Thr Asn Phe Phe His Glu Ser Gly Leu Glu
            900                 905                 910
Ala His Val Ala Leu Lys Pro Gly Lys Leu Lys Phe Ile Ile Pro Ser
        915                 920                 925
Pro Lys Arg Pro Val Lys Leu Leu Ser Gly Gly Asn Thr Leu His Leu
    930                 935                 940
Val Ser Thr Thr Lys Thr Glu Val Ile Pro Pro Leu Ile Glu Asn Arg
945                 950                 955                 960
Gln Ser Trp Ser Val Cys Lys Gln Val Phe Pro Gly Leu Asn Tyr Cys
                965                 970                 975
Thr Ser Gly Ala Tyr Ser Asn Ala Ser Ser Thr Asp Ser Ala Ser Tyr
            980                 985                 990
Tyr Pro Leu Thr Gly Asp Thr Arg Leu Glu Leu Glu Leu Arg Pro Thr
        995                 1000                1005
Gly Glu Ile Glu Gln Tyr Ser Val Ser Ala Thr Tyr Glu Leu Gln Arg
    1010                1015                1020
Glu Asp Arg Ala Leu Val Asp Thr Leu Lys Phe Val Thr Gln Ala Glu
1025                1030                1035                1040
Gly Ala Lys Gln Thr Glu Ala Thr Met Thr Phe Lys Tyr Asn Arg Gln
                1045                1050                1055
Ser Met Thr Leu Ser Ser Glu Val Gln Ile Pro Asp Phe Asp Val Asp
            1060                1065                1070
Leu Gly Thr Ile Leu Arg Val Asn Asp Glu Ser Thr Glu Gly Lys Thr
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        1075                1080                1085
Ser Tyr Arg Leu Thr Leu Asp Ile Gln Asn Lys Lys Ile Thr Glu Val
    1090                1095                1100
Ala Leu Met Gly His Leu Ser Cys Asp Thr Lys Glu Glu Arg Lys Ile
1105                1110                1115                1120
Lys Gly Val Ile Ser Ile Pro Arg Leu Gln Ala Glu Ala Arg Ser Glu
                1125                1130                1135
Ile Leu Ala His Trp Ser Pro Ala Lys Leu Leu Leu Gln Met Asp Ser
            1140                1145                1150
Ser Ala Thr Ala Tyr Gly Ser Thr Val Ser Lys Arg Val Ala Trp His
        1155                1160                1165
Tyr Asp Glu Glu Lys Ile Glu Phe Glu Trp Asn Thr Gly Thr Asn Val
    1170                1175                1180
Asp Thr Lys Lys Met Thr Ser Asn Phe Pro Val Asp Leu Ser Asp Tyr
1185                1190                1195                1200
Pro Lys Ser Leu His Met Tyr Ala Asn Arg Leu Leu Asp His Arg Val
                1205                1210                1215
Pro Gln Thr Asp Met Thr Phe Arg His Val Gly Ser Lys Leu Ile Val
            1220                1225                1230
Ala Met Ser Ser Trp Leu Gln Lys Ala Ser Gly Ser Leu Pro Tyr Thr
        1235                1240                1245
Gln Thr Leu Gln Asp His Leu Asn Ser Leu Lys Glu Phe Asn Leu Gln
    1250                1255                1260
Asn Met Gly Leu Pro Asp Phe His Ile Pro Glu Asn Leu Phe Leu Lys
1265                1270                1275                1280
Ser Asp Gly Arg Val Lys Tyr Thr Leu Asn Lys Asn Ser Leu Lys Ile
                1285                1290                1295
Glu Ile Pro Leu Pro Phe Gly Gly Lys Ser Ser Arg Asp Leu Lys Met
            1300                1305                1310
Leu Glu Thr Val Arg Thr Pro Ala Leu His Phe Lys Ser Val Gly Phe
        1315                1320                1325
His Leu Pro Ser Arg Glu Phe Gln Val Pro Thr Phe Thr Ile Pro Lys
    1330                1335                1340
Leu Tyr Gln Leu Gln Val Pro Leu Leu Gly Val Leu Asp Leu Ser Thr
1345                1350                1355                1360
Asn Val Tyr Ser Asn Leu Tyr Asn Trp Ser Ala Ser Tyr Ser Gly Gly
                1365                1370                1375
Asn Thr Ser Thr Asp His Phe Ser Leu Arg Ala Arg Tyr His Met Lys
            1380                1385                1390
Ala Asp Ser Val Val Asp Leu Leu Ser Tyr Asn Val Gln Gly Ser Gly
        1395                1400                1405
Glu Thr Thr Tyr Asp His Lys Asn Thr Phe Thr Leu Ser Cys Asp Gly
    1410                1415                1420
Ser Leu Arg His Lys Phe Leu Asp Ser Asn Ile Lys Phe Ser His Val
1425                1430                1435                1440
Glu Lys Leu Gly Asn Asn Pro Val Ser Lys Gly Leu Leu Ile Phe Asp
                1445                1450                1455
Ala Ser Ser Ser Trp Gly Pro Gln Met Ser Ala Ser Val His Leu Asp
            1460                1465                1470
Ser Lys Lys Lys Gln His Leu Phe Val Lys Glu Val Lys Ile Asp Gly
        1475                1480                1485
Gln Phe Arg Val Ser Ser Phe Tyr Ala Lys Gly Thr Tyr Gly Leu Ser
    1490                1495                1500
Cys Gln Arg Asp Pro Asn Thr Gly Arg Leu Asn Gly Glu Ser Asn Leu
1505                1510                1515                1520
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Arg Phe Asn Ser Ser Tyr Leu Gln Gly Thr Asn Gln Ile Thr Gly Arg
1525 1530 1535
Tyr Glu Asp Gly Thr Leu Ser Leu Thr Ser Thr Ser Asp Leu Gln Ser
1540 1545 1550
Gly Ile Ile Lys Asn Thr Ala Ser Leu Lys Tyr Glu Asn Tyr Glu Leu
1555 1560 1565
Thr Leu Lys Ser Asp Thr Asn Gly Lys Tyr Lys Asn Phe Ala Thr Ser
1570 1575 1580
Asn Lys Met Asp Met Thr Phe Ser Lys Gln Asn Ala Leu Leu Arg Ser
1585 1590 1595 1600
Glu Tyr Gln Ala Asp Tyr Glu Ser Leu Arg Phe Phe Ser Leu Leu Ser
1605 1610 1615
Gly Ser Leu Asn Ser His Gly Leu Glu Leu Asn Ala Asp Ile Leu Gly
1620 1625 1630
Thr Asp Lys Ile Asn Ser Gly Ala His Lys Ala Thr Leu Arg Ile Gly
1635 1640 1645
Gln Asp Gly Ile Ser Thr Ser Ala Thr Thr Asn Leu Lys Cys Ser Leu
1650 1655 1660
Leu Val Leu Glu Asn Glu Leu Asn Ala Glu Leu Gly Leu Ser Gly Ala
1665 1670 1675 1680
Ser Met Lys Leu Thr Thr Asn Gly Arg Phe Arg Glu His Asn Ala Lys
1685 1690 1695
Phe Ser Leu Asp Gly Lys Ala Ala Leu Thr Glu Leu Ser Leu Gly Ser
1700 1705 1710
Ala Tyr Gln Ala Met Ile Leu Gly Val Asp Ser Lys Asn Ile Phe Asn
1715 1720 1725
Phe Lys Val Ser Gln Glu Gly Leu Lys Leu Ser Asn Asp Met Met Gly
1730 1735 1740
Ser Tyr Ala Glu Met Lys Phe Asp His Thr Asn Ser Leu Asn Ile Ala
1745 1750 1755 1760
Gly Leu Ser Leu Asp Phe Ser Ser Lys Leu Asp Asn Ile Tyr Ser Ser
1765 1770 1775
Asp Lys Phe Tyr Lys Gln Thr Val Asn Leu Gln Leu Gln Pro Tyr Ser
1780 1785 1790
Leu Val Thr Thr Leu Asn Ser Asp Leu Lys Tyr Asn Ala Leu Asp Leu
1795 1800 1805
Thr Asn Asn Gly Lys Leu Arg Leu Glu Pro Leu Lys Leu His Val Ala
1810 1815 1820
Gly Asn Leu Lys Gly Ala Tyr Gln Asn Asn Glu Ile Lys His Ile Tyr
1825 1830 1835 1840
Ala Ile Ser Ser Ala Ala Leu Ser Ala Ser Tyr Lys Ala Asp Thr Val
1845 1850 1855
Ala Lys Val Gln Gly Val Glu Phe Ser His Arg Leu Asn Thr Asp Ile
1860 1865 1870
Ala Gly Leu Ala Ser Ala Ile Asp Met Ser Thr Asn Tyr Asn Ser Asp
1875 1880 1885
Ser Leu His Phe Ser Asn Val Phe Arg Ser Val Met Ala Pro Phe Thr
1890 1895 1900
Met Thr Ile Asp Ala His Thr Asn Gly Asn Gly Lys Leu Ala Leu Trp
1905 1910 1915 1920
Gly Glu His Thr Gly Gln Leu Tyr Ser Lys Phe Leu Leu Lys Ala Glu
1925 1930 1935
Pro Leu Ala Phe Thr Phe Ser His Asp Tyr Lys Gly Ser Thr Ser His
1940 1945 1950
His Leu Val Ser Arg Lys Ser Ile Ser Ala Ala Leu Glu His Lys Val

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        1955                1960                1965
Ser Ala Leu Leu Thr Pro Ala Glu Gln Thr Gly Thr Trp Lys Leu Lys
    1970                1975                1980
Thr Gln Phe Asn Asn Asn Glu Tyr Ser Gln Asp Leu Asp Ala Tyr Asn
1985                1990                1995                2000
Thr Lys Asp Lys Ile Gly Val Glu Leu Thr Gly Arg Thr Leu Ala Asp
                2005                2010                2015
Leu Thr Leu Leu Asp Ser Pro Ile Lys Val Pro Leu Leu Leu Ser Glu
            2020                2025                2030
Pro Ile Asn Ile Ile Asp Ala Leu Glu Met Arg Asp Ala Val Glu Lys
        2035                2040                2045
Pro Gln Glu Phe Thr Ile Val Ala Phe Val Lys Tyr Asp Lys Asn Gln
    2050                2055                2060
Asp Val His Ser Ile Asn Leu Pro Phe Phe Glu Thr Leu Gln Glu Tyr
2065                2070                2075                2080
Phe Glu Arg Asn Arg Gln Thr Ile Ile Val Val Leu Glu Asn Val Gln
                2085                2090                2095
Arg Asn Leu Lys His Ile Asn Ile Asp Gln Phe Val Arg Lys Tyr Arg
            2100                2105                2110
Ala Ala Leu Gly Lys Leu Pro Gln Gln Ala Asn Asp Tyr Leu Asn Ser
        2115                2120                2125
Phe Asn Trp Glu Arg Gln Val Ser His Ala Lys Glu Lys Leu Thr Ala
    2130                2135                2140
Leu Thr Lys Lys Tyr Arg Ile Thr Glu Asn Asp Ile Gln Ile Ala Leu
2145                2150                2155                2160
Asp Asp Ala Lys Ile Asn Phe Asn Glu Lys Leu Ser Gln Leu Gln Thr
                2165                2170                2175
Tyr Met Ile Gln Phe Asp Gln Tyr Ile Lys Asp Ser Tyr Asp Leu His
            2180                2185                2190
Asp Leu Lys Ile Ala Ile Ala Asn Ile Ile Asp Glu Ile Ile Glu Lys
        2195                2200                2205
Leu Lys Ser Leu Asp Glu His Tyr His Ile Arg Val Asn Leu Val Lys
    2210                2215                2220
Thr Ile His Asp Leu His Leu Phe Ile Glu Asn Ile Asp Phe Asn Lys
2225                2230                2235                2240
Ser Gly Ser Ser Thr Ala Ser Trp Ile Gln Asn Val Asp Thr Lys Tyr
                2245                2250                2255
Gln Ile Arg Ile Gln Ile Gln Glu Lys Leu Gln Gln Leu Lys Arg His
            2260                2265                2270
Ile Gln Asn Ile Asp Ile Gln His Leu Ala Gly Lys Leu Lys Gln His
        2275                2280                2285
Ile Glu Ala Ile Asp Val Arg Val Leu Leu Asp Gln Leu Gly Thr Thr
    2290                2295                2300
Ile Ser Phe Glu Arg Ile Asn Asp Val Leu Glu His Val Lys His Phe
2305                2310                2315                2320
Val Ile Asn Leu Ile Gly Asp Phe Glu Val Ala Glu Lys Ile Asn Ala
                2325                2330                2335
Phe Arg Ala Lys Val His Glu Leu Ile Glu Arg Tyr Glu Val Asp Gln
            2340                2345                2350
Gln Ile Gln Val Leu Met Asp Lys Leu Val Glu Leu Ala His Gln Tyr
        2355                2360                2365
Lys Leu Lys Glu Thr Ile Gln Lys Leu Ser Asn Val Leu Gln Gln Val
    2370                2375                2380
Lys Ile Lys Asp Tyr Phe Glu Lys Leu Val Gly Phe Ile Asp Asp Ala
2385                2390                2395                2400
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Val Lys Lys Leu Asn Glu Leu Ser Phe Lys Thr Phe Ile Glu Asp Val
                2405                2410                2415
Asn Lys Phe Leu Asp Met Leu Ile Lys Lys Leu Lys Ser Phe Asp Tyr
            2420                2425                2430
His Gln Phe Val Asp Glu Thr Asn Asp Lys Ile Arg Glu Val Thr Gln
        2435                2440                2445
Arg Leu Asn Gly Glu Ile Gln Ala Leu Glu Leu Pro Gln Lys Ala Glu
    2450                2455                2460
Ala Leu Lys Leu Phe Leu Glu Glu Thr Lys Ala Thr Val Ala Val Tyr
2465                2470                2475                2480
Leu Glu Ser Leu Gln Asp Thr Lys Ile Thr Leu Ile Ile Asn Trp Leu
                2485                2490                2495
Gln Glu Ala Leu Ser Ser Ala Ser Leu Ala His Met Lys Ala Lys Phe
            2500                2505                2510
Arg Glu Thr Leu Glu Asp Thr Arg Asp Arg Met Tyr Gln Met Asp Ile
        2515                2520                2525
Gln Gln Glu Leu Gln Arg Tyr Leu Ser Leu Val Gly Gln Val Tyr Ser
    2530                2535                2540
Thr Leu Val Thr Tyr Ile Ser Asp Trp Trp Thr Leu Ala Ala Lys Asn
2545                2550                2555                2560
Leu Thr Asp Phe Ala Glu Gln Tyr Ser Ile Gln Asp Trp Ala Lys Arg
                2565                2570                2575
Met Lys Ala Leu Val Glu Gln Gly Phe Thr Val Pro Glu Ile Lys Thr
            2580                2585                2590
Ile Leu Gly Thr Met Pro Ala Phe Glu Val Ser Leu Gln Ala Leu Gln
        2595                2600                2605
Lys Ala Thr Phe Gln Thr Pro Asp Phe Ile Val Pro Leu Thr Asp Leu
    2610                2615                2620
Arg Ile Pro Ser Val Gln Ile Asn Phe Lys Asp Leu Lys Asn Ile Lys
2625                2630                2635                2640
Ile Pro Ser Arg Phe Ser Thr Pro Glu Phe Thr Ile Leu Asn Thr Phe
                2645                2650                2655
His Ile Pro Ser Phe Thr Ile Asp Phe Val Glu Met Lys Val Lys Ile
            2660                2665                2670
Ile Arg Thr Ile Asp Gln Met Leu Asn Ser Glu Leu Gln Trp Pro Val
        2675                2680                2685
Pro Asp Ile Tyr Leu Arg Asp Leu Lys Val Glu Asp Ile Pro Leu Ala
    2690                2695                2700
Arg Ile Thr Leu Pro Asp Phe Arg Leu Pro Glu Ile Ala Ile Pro Glu
2705                2710                2715                2720
Phe Ile Ile Pro Thr Leu Asn Leu Asn Asp Phe Gln Val Pro Asp Leu
                2725                2730                2735
His Ile Pro Glu Phe Gln Leu Pro His Ile Ser His Thr Ile Glu Val
            2740                2745                2750
Pro Thr Phe Gly Lys Leu Tyr Ser Ile Leu Lys Ile Gln Ser Pro Leu
        2755                2760                2765
Phe Thr Leu Asp Ala Asn Ala Asp Ile Gly Asn Gly Thr Thr Ser Ala
    2770                2775                2780
Asn Glu Ala Gly Ile Ala Ala Ser Ile Thr Ala Lys Gly Glu Ser Lys
2785                2790                2795                2800
Leu Glu Val Leu Asn Phe Asp Phe Gln Ala Asn Ala Gln Leu Ser Asn
                2805                2810                2815
Pro Lys Ile Asn Pro Leu Ala Leu Lys Glu Ser Val Lys Phe Ser Ser
            2820                2825                2830
Lys Tyr Leu Arg Thr Glu His Gly Ser Glu Met Leu Phe Phe Gly Asn
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        2835                2840                2845
Ala Ile Glu Gly Lys Ser Asn Thr Val Ala Ser Leu His Thr Glu Lys
    2850                2855                2860
Asn Thr Leu Glu Leu Ser Asn Gly Val Ile Val Lys Ile Asn Asn Gln
2865                2870                2875                2880
Leu Thr Leu Asp Ser Asn Thr Lys Tyr Phe His Lys Leu Asn Ile Pro
                2885                2890                2895
Lys Leu Asp Phe Ser Ser Gln Ala Asp Leu Arg Asn Glu Ile Lys Thr
            2900                2905                2910
Leu Leu Lys Ala Gly His Ile Ala Trp Thr Ser Ser Gly Lys Gly Ser
        2915                2920                2925
Trp Lys Trp Ala Cys Pro Arg Phe Ser Asp Glu Gly Thr His Glu Ser
    2930                2935                2940
Gln Ile Ser Phe Thr Ile Glu Gly Pro Leu Thr Ser Phe Gly Leu Ser
2945                2950                2955                2960
Asn Lys Ile Asn Ser Lys His Leu Arg Val Asn Gln Asn Leu Val Tyr
                2965                2970                2975
Glu Ser Gly Ser Leu Asn Phe Ser Lys Leu Glu Ile Gln Ser Gln Val
            2980                2985                2990
Asp Ser Gln His Val Gly His Ser Val Leu Thr Ala Lys Gly Met Ala
        2995                3000                3005
Leu Phe Gly Glu Gly Lys Ala Glu Phe Thr Gly Arg His Asp Ala His
    3010                3015                3020
Leu Asn Gly Lys Val Ile Gly Thr Leu Lys Asn Ser Leu Phe Phe Ser
3025                3030                3035                3040
Ala Gln Pro Phe Glu Ile Thr Ala Ser Thr Asn Asn Glu Gly Asn Leu
                3045                3050                3055
Lys Val Arg Phe Pro Leu Arg Leu Thr Gly Lys Ile Asp Phe Leu Asn
            3060                3065                3070
Asn Tyr Ala Leu Phe Leu Ser Pro Ser Ala Gln Gln Ala Ser Trp Gln
        3075                3080                3085
Val Ser Ala Arg Phe Asn Gln Tyr Lys Tyr Asn Gln Asn Phe Ser Ala
    3090                3095                3100
Gly Asn Asn Glu Asn Ile Met Glu Ala His Val Gly Ile Asn Gly Glu
3105                3110                3115                3120
Ala Asn Leu Asp Phe Leu Asn Ile Pro Leu Thr Ile Pro Glu Met Arg
                3125                3130                3135
Leu Pro Tyr Thr Ile Ile Thr Thr Pro Pro Leu Lys Asp Phe Ser Leu
            3140                3145                3150
Trp Glu Lys Thr Gly Leu Lys Glu Phe Leu Lys Thr Thr Lys Gln Ser
        3155                3160                3165
Phe Asp Leu Ser Val Lys Ala Gln Tyr Lys Lys Asn Lys His Arg His
    3170                3175                3180
Ser Ile Thr Asn Pro Leu Ala Val Leu Cys Glu Phe Ile Ser Gln Ser
3185                3190                3195                3200
Ile Lys Ser Phe Asp Arg His Phe Glu Lys Asn Arg Asn Asn Ala Leu
                3205                3210                3215
Asp Phe Val Thr Lys Ser Tyr Asn Glu Thr Lys Ile Lys Phe Asp Lys
            3220                3225                3230
Tyr Lys Ala Glu Lys Ser His Asp Glu Leu Pro Arg Thr Phe Gln Ile
        3235                3240                3245
Pro Gly Tyr Thr Val Pro Val Val Asn Val Glu Val Ser Pro Phe Thr
    3250                3255                3260
Ile Glu Met Ser Ala Phe Gly Tyr Val Phe Pro Lys Ala Val Ser Met
3265                3270                3275                3280
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Pro Ser Phe Ser Ile Leu Gly Ser Asp Val Arg Val Pro Ser Tyr Thr
3285 3290 3295
Leu Ile Leu Pro Ser Leu Glu Leu Pro Val Leu His Val Pro Arg Asn
3300 3305 3310
Leu Lys Leu Ser Leu Pro Asp Phe Lys Glu Leu Cys Thr Ile Ser His
3315 3320 3325
Ile Phe Ile Pro Ala Met Gly Asn Ile Thr Tyr Asp Phe Ser Phe Lys
3330 3335 3340
Ser Ser Val Ile Thr Leu Asn Thr Asn Ala Glu Leu Phe Asn Gln Ser
3345 3350 3355 3360
Asp Ile Val Ala His Leu Leu Ser Ser Ser Ser Ser Val Ile Asp Ala
3365 3370 3375
Leu Gln Tyr Lys Leu Glu Gly Thr Thr Arg Leu Thr Arg Lys Arg Gly
3380 3385 3390
Leu Lys Leu Ala Thr Ala Leu Ser Leu Ser Asn Lys Phe Val Glu Gly
3395 3400 3405
Ser His Asn Ser Thr Val Ser Leu Thr Thr Lys Asn Met Glu Val Ser
3410 3415 3420
Val Ala Thr Thr Thr Lys Ala Gln Ile Pro Ile Leu Arg Met Asn Phe
3425 3430 3435 3440
Lys Gln Glu Leu Asn Gly Asn Thr Lys Ser Lys Pro Thr Val Ser Ser
3445 3450 3455
Ser Met Glu Phe Lys Tyr Asp Phe Asn Ser Ser Met Leu Tyr Ser Thr
3460 3465 3470
Ala Lys Gly Ala Val Asp His Lys Leu Ser Leu Glu Ser Leu Thr Ser
3475 3480 3485
Tyr Phe Ser Ile Glu Ser Ser Thr Lys Gly Asp Val Lys Gly Ser Val
3490 3495 3500
Leu Ser Arg Glu Tyr Ser Gly Thr Ile Ala Ser Glu Ala Asn Thr Tyr
3505 3510 3515 3520
Leu Asn Ser Lys Ser Thr Arg Ser Ser Val Lys Leu Gln Gly Thr Ser
3525 3530 3535
Lys Ile Asp Asp Ile Trp Asn Leu Glu Val Lys Glu Asn Phe Ala Gly
3540 3545 3550
Glu Ala Thr Leu Gln Arg Ile Tyr Ser Leu Trp Glu His Ser Thr Lys
3555 3560 3565
Asn His Leu Gln Leu Glu Gly Leu Phe Phe Thr Asn Gly Glu His Thr
3570 3575 3580
Ser Lys Ala Thr Leu Glu Leu Ser Pro Trp Gln Met Ser Ala Leu Val
3585 3590 3595 3600
Gln Val His Ala Ser Gln Pro Ser Ser Phe His Asp Phe Pro Asp Leu
3605 3610 3615
Gly Gln Glu Val Ala Leu Asn Ala Asn Thr Lys Asn Gln Lys Ile Arg
3620 3625 3630
Trp Lys Asn Glu Val Arg Ile His Ser Gly Ser Phe Gln Ser Gln Val
3635 3640 3645
Glu Leu Ser Asn Asp Gln Glu Lys Ala His Leu Asp Ile Ala Gly Ser
3650 3655 3660
Leu Glu Gly His Leu Arg Phe Leu Lys Asn Ile Ile Leu Pro Val Tyr
3665 3670 3675 3680
Asp Lys Ser Leu Trp Asp Phe Leu Lys Leu Asp Val Thr Thr Ser Ile
3685 3690 3695
Gly Arg Arg Gln His Leu Arg Val Ser Thr Ala Phe Val Tyr Thr Lys
3700 3705 3710
Asn Pro Asn Gly Tyr Ser Phe Ser Ile Pro Val Lys Val Leu Ala Asp

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        3715                3720                3725
Lys Phe Ile Ile Pro Gly Leu Lys Leu Asn Asp Leu Asn Ser Val Leu
    3730                3735                3740
Val Met Pro Thr Phe His Val Pro Phe Thr Asp Leu Gln Val Pro Ser
3745                3750                3755                3760
Cys Lys Leu Asp Phe Arg Glu Ile Gln Ile Tyr Lys Lys Leu Arg Thr
                3765                3770                3775
Ser Ser Phe Ala Leu Asn Leu Pro Thr Leu Pro Glu Val Lys Phe Pro
            3780                3785                3790
Glu Val Asp Val Leu Thr Lys Tyr Ser Gln Pro Glu Asp Ser Leu Ile
        3795                3800                3805
Pro Phe Phe Glu Ile Thr Val Pro Glu Ser Gln Leu Thr Val Ser Gln
    3810                3815                3820
Phe Thr Leu Pro Lys Ser Val Ser Asp Gly Ile Ala Ala Leu Asp Leu
3825                3830                3835                3840
Asn Ala Val Ala Asn Lys Ile Ala Asp Phe Glu Leu Pro Thr Ile Ile
                3845                3850                3855
Val Pro Glu Gln Thr Ile Glu Ile Pro Ser Ile Lys Phe Ser Val Pro
            3860                3865                3870
Ala Gly Ile Ala Ile Pro Ser Phe Gln Ala Leu Thr Ala Arg Phe Glu
        3875                3880                3885
Val Asp Ser Pro Val Tyr Asn Ala Thr Trp Ser Ala Ser Leu Lys Asn
    3890                3895                3900
Lys Ala Asp Tyr Val Glu Thr Val Leu Asp Ser Thr Cys Ser Ser Thr
3905                3910                3915                3920
Val Gln Phe Leu Glu Tyr Glu Leu Asn Val Leu Gly Thr His Lys Ile
                3925                3930                3935
Glu Asp Gly Thr Leu Ala Ser Lys Thr Lys Gly Thr Phe Ala His Arg
            3940                3945                3950
Asp Phe Ser Ala Glu Tyr Glu Glu Asp Gly Lys Tyr Glu Gly Leu Gln
        3955                3960                3965
Glu Trp Glu Gly Lys Ala His Leu Asn Ile Lys Ser Pro Ala Phe Thr
    3970                3975                3980
Asp Leu His Leu Arg Tyr Gln Lys Asp Lys Lys Gly Ile Ser Thr Ser
3985                3990                3995                4000
Ala Ala Ser Pro Ala Val Gly Thr Val Gly Met Asp Met Asp Glu Asp
                4005                4010                4015
Asp Asp Phe Ser Lys Trp Asn Phe Tyr Tyr Ser Pro Gln Ser Ser Pro
            4020                4025                4030
Asp Lys Lys Leu Thr Ile Phe Lys Thr Glu Leu Arg Val Arg Glu Ser
        4035                4040                4045
Asp Glu Glu Thr Gln Ile Lys Val Asn Trp Glu Glu Glu Ala Ala Ser
    4050                4055                4060
Gly Leu Leu Thr Ser Leu Lys Asp Asn Val Pro Lys Ala Thr Gly Val
4065                4070                4075                4080
Leu Tyr Asp Tyr Val Asn Lys Tyr His Trp Glu His Thr Gly Leu Thr
                4085                4090                4095
Leu Arg Glu Val Ser Ser Lys Leu Arg Arg Asn Leu Gln Asp His Ala
            4100                4105                4110
Glu Trp Val Tyr Gln Gly Ala Ile Arg Glu Ile Asp Asp Ile Asp Glu
        4115                4120                4125
Arg Phe Gln Lys Gly Ala Ser Gly Thr Thr Gly Thr Tyr Gln Glu Trp
    4130                4135                4140
Lys Asp Lys Ala Gln Asn Leu Tyr Gln Glu Leu Leu Thr Gln Glu Gly
4145                4150                4155                4160
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Gln Ala Ser Phe Gln Gly Leu Lys Asp Asn Val Phe Asp Gly Leu Val
                4165                4170                4175
Arg Val Thr Gln Glu Phe His Met Lys Val Lys His Leu Ile Asp Ser
            4180                4185                4190
Leu Ile Asp Phe Leu Asn Phe Pro Arg Phe Gln Phe Pro Gly Lys Pro
        4195                4200                4205
Gly Ile Tyr Thr Arg Glu Glu Leu Cys Thr Met Phe Ile Arg Glu Val
    4210                4215                4220
Gly Thr Val Leu Ser Gln Val Tyr Ser Lys Val His Asn Gly Ser Glu
4225                4230                4235                4240
Ile Leu Phe Ser Tyr Phe Gln Asp Leu Val Ile Thr Leu Pro Phe Glu
                4245                4250                4255
Leu Arg Lys His Lys Leu Ile Asp Val Ile Ser Met Tyr Arg Glu Leu
            4260                4265                4270
Leu Lys Asp Leu Ser Lys Glu Ala Gln Glu Val Phe Lys Ala Ile Gln
        4275                4280                4285
Ser Leu Lys Thr Thr Glu Val Leu Arg Asn Leu Gln Asp Leu Leu Gln
    4290                4295                4300
Phe Ile Phe Gln Leu Ile Glu Asp Asn Ile Lys Gln Leu Lys Glu Met
4305                4310                4315                4320
Lys Phe Thr Tyr Leu Ile Asn Tyr Ile Gln Asp Glu Ile Asn Thr Ile
                4325                4330                4335
Phe Asn Asp Tyr Ile Pro Tyr Val Phe Lys Leu Leu Lys Glu Asn Leu
            4340                4345                4350
Cys Leu Asn Leu His Lys Phe Asn Glu Phe Ile Gln Asn Glu Leu Gln
        4355                4360                4365
Glu Ala Ser Gln Glu Leu Gln Gln Ile His Gln Tyr Ile Met Ala Leu
    4370                4375                4380
Arg Glu Glu Tyr Phe Asp Pro Ser Ile Val Gly Trp Thr Val Lys Tyr
4385                4390                4395                4400
Tyr Glu Leu Glu Glu Lys Ile Val Ser Leu Ile Lys Asn Leu Leu Val
                4405                4410                4415
Ala Leu Lys Asp Phe His Ser Glu Tyr Ile Val Ser Ala Ser Asn Phe
            4420                4425                4430
Thr Ser Gln Leu Ser Ser Gln Val Glu Gln Phe Leu His Arg Asn Ile
        4435                4440                4445
Gln Glu Tyr Leu Ser Ile Leu Thr Asp Pro Asp Gly Lys Gly Lys Glu
    4450                4455                4460
Lys Ile Ala Glu Leu Ser Ala Thr Ala Gln Glu Ile Ile Lys Ser Gln
4465                4470                4475                4480
Ala Ile Ala Thr Lys Lys Ile Ile Ser Asp Tyr His Gln Gln Phe Arg
                4485                4490                4495
Tyr Lys Leu Gln Asp Phe Ser Asp Gln Leu Ser Asp Tyr Tyr Glu Lys
            4500                4505                4510
Phe Ile Ala Glu Ser Lys Arg Leu Ile Asp Leu Ser Ile Gln Asn Tyr
        4515                4520                4525
His Thr Phe Leu Ile Tyr Ile Thr Glu Leu Leu Lys Lys Leu Gln Ser
    4530                4535                4540
Thr Thr Val Met Asn Pro Tyr Met Lys Leu Ala Pro Gly Glu Leu Thr
4545                4550                4555                4560
Ile Ile Leu
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<211> 14070

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/17908

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 43/04; C07H 21/04; C12N 15/09, 15/11, 15/85

US CL :435/440, 455, 471, 490; 514/44; 536/23.1, 23.5, 24.1, 24.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/440, 455, 471, 490; 514/44; 536/23.1, 23.5, 24.1, 24.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, EMBASE, MEDLINE, DERWENT

search terms: apo? ldl? low density lipoprotein? cholesterol? atherosclero? clathrin?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	US 5,736,157 A (WILLIAMS) 07 April 1998, see entire document.	1-4, 7-9, 24-50
A,E	US 4,772,549 A (FROSSARD) 20 September 1988, see entire document.	1-4, 7-9, 24-50

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 22 DECEMBER 1998	Date of mailing of the international search report 05 FEB 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer NANCY J. DEGEN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/17908

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-6, 10-23, 51-52
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.